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BRITISH PHARMACOLOGICAL SOCIETY

WINTER MEETING, 7th to 8th JANUARY, 1960,

AT THE SCHOOL OF PHARMACY, BRUNSWICK SQUARE, LONDON

COMMUNICATIONS

1. **G. A. Feigen** and **E. M. Vaughan Williams** (*Department of Pharmacology, Oxford*).
Electrophysiological evidence for the release of histamine during an antigen-antibody reaction in isolated guinea-pig auricles.

2. **K. R. Butterworth** and **Monica Mann** (*Departments of Pharmacology, St. Mary's Hospital Medical School, London, W.2, and School of Pharmacy, London, W.C.*1).
Variations in the catechol amine content of the adrenal glands of normal cats.

3. **J. R. Hodges** and **Joan Vernikos** (*Department of Pharmacology, Royal Free Hospital School of Medicine, London, W.C.*1).
The role of corticoids in regulating pituitary adrenocorticotrophic activity.

4. **J. M. Barnes** (*Toxicology Research Unit, Medical Research Council Laboratories, Carshalton, Surrey*).
The toxicity of acrylamide.

5. **W. H. H. Andrews** and **I. del Rio Lozano** (*Department of Pharmacology, St. Mary's Hospital Medical School, London, W.2*).
The effect of the route of administration of some drugs upon their rate of destruction by the liver.

6. **K. Ahmed** and **J. J. Lewis** (*Experimental Pharmacology, The University, Glasgow, W.2*).
Pharmacology of Petalin ; a convulsant alkaloid.

7. **D. R. Davies, P. Holland** and **M. J. Rumens** (*C.D.E.E., Porton Down, Salisbury, Wilts.*).
The relationship between the chemical structure and neurotoxicity of alkyl organophosphorus compounds. (*Precirculated.*)

8. **Z. M. Bacq** and **J. Renson** (*Laboratoire de Pathologie et Thérapeutique Générales, Liége*).
The inhibitors of adrenaline and noradrenaline inactivation. (*Precirculated.*)

9. **R. Schneider, Hilary Bishop, B. Shaw** and **A. C. Frazer** (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham*, 15).
Further observations on the mechanism of the inhibitory effect of gluten on isolated intestine.

10. **T. J. Sullivan** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).
The effect of some antibiotics on the amount of 5-hydroxytryptamine in the small intestine and spleen of mice and rats.

11. **S. E. Smith** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).
Some actions of α-methyldopa.

12. **I. Laszlo** (*introduced by* **R. S. Stacey**) (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.*1).
Observations on the estimation of substance P in mouse brain. (*Precirculated.*)

13. **J. H. Mackintosh** and **M. R. A. Chance** (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham*, 15).
Heredity and environment in the control of the variance of barbiturate anaesthesia.

14. **W. G. Smith** (*Department of Pharmacy, Sunderland Technical College*).
The anti-anaphylactic activity of ethanolamine and choline. (*Precirculated.*)

15. **J. J. Reuse** (*Laboratoire de Pharmacodynamie et de Thérapeutique, Université de Bruxelles*).
The effects of reserpine upon convulsions in rabbits.

16. **T. B. B. Crawford** and **D. F. Sharman** (*Department of Pharmacology, University of Edinburgh*).

Some observations on the methods of estimating 5-hydroxytryptamine in brain.

17. **E. H. Leach** and **P. A. Nasmyth** (*Department of Physiology, Oxford, and Department of Pharmacology, St. Mary's Hospital Medical School, London, W.*2).

Potentiation of the effects of tyramine by choline xylyl ether.

18. **R. W. Foster** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).

The action of sympathomimetic drugs on the isolated guinea-pig tracheal muscle.

19. **A. B. Wilson** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).

A method of analysing the inhibitory effects of sympathomimetic amines.

20. **G. D. H. Leach** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).

The effect of central nervous stimulants and depressants on the movement of the transmurally stimulated guinea-pig ileum.

21. **L. C. Blaber** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

The antagonistic action of ambenonium and its methoxy analogue to competitive and depolarizing neuromuscular blocking drugs. (*Precirculated.*)

22. **J. A. Holgate** and **B. T. Warner** (*Department of Pharmacological Research, Parke, Davis & Co., Hounslow, Middlesex*).

Potency of antihistamines *in vivo* as assessed by guinea-pig bronchiolar resistance. (*Precirculated.*)

23. **H. O. J. Collier** and **Patricia G. Shorley** (*Department of Pharmacological Research, Parke, Davis & Co., Hounslow, Middlesex*).

Effects of anti-rheumatic agents on some responses of guinea-pigs to bradykinin. (*Precirculated.*)

24. **G. V. R. Born** and **Janet Bricknell** (*Nuffield Institute for Medical Research, Oxford*).

5-Hydroxytryptamine in smooth muscle and connective tissue.

25. **B. Uvnäs** (*Farmakologiska Institutionen, Karolinska Institutet, Stockholm*, 60).

Histamine, slow reacting substance (SRS), and mast cell degranulation.

DEMONSTRATIONS

1. **Z. M. Bacq** and **J. Renson** (*Laboratoire de Pathologie et Thérapeutique Générales, Liége*).

Sensitization to adrenaline and adrenergic nerve stimulation by metanephrine. (*Precirculated.*)

2. **E. Letley** and **G. Batterson** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, University of London King's College, London, W.C.*2).

The changes in the flow of blood in denervated rat hind-limb muscles in relation to the inhibition by adrenaline and other substances of acetylcholine-produced contractures.

3. **P. S. J. Spencer** (*introduced by* **G. B. West**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

The effect of the thyroid on the histamine and 5-hydroxytryptamine toxicities in the rat and mouse. (*Precirculated.*)

4. **A. M. Barrett** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Some factors affecting blood levels of corticotrophin.

5. **Shirley A. P. Price** (*introduced by* **G. B. West**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

The estimation of, and the effects of drugs on, the 5-hydroxytryptophan decarboxylase in rat brain.

6. **G. B. West** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Indole derivatives in tomatoes.

7. **J. M. Telford** (*introduced by* **G. B. West**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

The action of some adrenal cortical hormones on histamine and 5-hydroxytryptamine metabolism. (*Precirculated.*)

8. **Eleanor J. Zaimis** (*Pharmacology Department, Royal Free Hospital School of Medicine, London, W.C.*1).

A simple test for an amphetamine-like action.

9. **P. A. Nasmyth** (*Department of Pharmacology, St. Mary's Hospital Medical School, London, W.*2).

The effect of choline xylyl ether on the chromaffine cells.

10. **Third Year Students** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Some student exercises.

11. **Eva Kovacs** and **G. A. H. Buttle** (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Effect of serum from tumour-bearing animals on the growth of a human tumour in rats.

12. **H. Ghaleb** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1, *and Royal Marsden Hospital, London, S.W.*3).

Binding of stilboestrol on to plasma proteins.

13. **A. W. Cuthbert** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, School of Pharmacy, London, W.C.*1).

Action of drugs on muscle in tissue culture.

Brit. J. Pharmacol. (1960), **15**, 4.

THE ACCUMULATION OF ISONIAZID IN TISSUES, AND THE COURSE OF EXPERIMENTAL TUBERCULOSIS

BY

J. VENULET, KRYSTYNA JAKIMOWSKA, M. JANOWIEC, AND ALICJA URBAŃSKA

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In an attempt to find agents enhancing the infiltration of isoniazid into tissues, the authors have investigated the effect of the drug on oedema due to injection of egg white into the rat paw, when it was given alone and in conjunction with histamine, antazoline, carbachol, chlorpromazine, and a pyrogen. Isoniazid increased the oedema. All the substances, except antazoline, increased the rate at which isoniazid disappeared from the blood, and they all favoured accumulation of the drug in tissues. Treatment of experimental tuberculosis with subthreshold doses of isoniazid, in conjunction with the above compounds, gave better results than when isoniazid was used alone.

Of the numerous factors affecting the final results of tuberculosis treatment, the accessibility of the drugs to the site of infection is of fundamental importance. The duration of the disease and the attendant protective and toxic reactions, as for example fibrosis and caseation, are serious impediments to the drug reaching the infected areas. The positive results obtained when the disease is treated with a potent antitubercular drug in conjunction with either corticotrophin or cortisone are explained by the paradoxically favourable effects of inhibition of some protective processes.

The experiments described in this paper were designed to determine the degree to which some antitubercular drugs and other substances might modify the inflammatory reaction and the permeability of tissues. Their value in treating experimental tuberculosis was also explored.

METHODS

The volume of extracellular fluid was measured in rabbits by the thiocyanate method (Odier, 1948), and the volume of circulating blood by the method of Griesbach (1921) and Heilmeyer (1929). Oedema of the rat paw was induced by injection into the paw of 0.05 ml. of egg white diluted 1 in 3 (Gross, 1950). Isoniazid concentrations were determined in rabbit blood and in rat lungs, liver, and kidney by the method of Deeb and Vitagliano (1955). In order to eliminate the opalescence in liver samples glycogen was hydrolysed with diastase.

Experimental tuberculosis was produced in albino mice weighing about 20 g. and in guinea-pigs weighing about 350 g. The mice were injected intravenously with 0.1 mg. of strain $H_{37}Rv$ and the guinea-pigs subcutaneously with 0.3 mg. of strain Sz. The drugs were administered subcutaneously daily from the day after inoculation until all the control animals had died. Then the surviving test animals were killed and the degree of the tubercular lesions was estimated microscopically. Three factors were determined with respect to the control group: (*a*) the death rate in each group, (*b*) the mean survival time, and (*c*) the index of tuberculosis.

The substances used in the experiments comprised: Isoniazid (isonicotinic acid hydrazide), histamine; antazoline (Antistine, Ciba), chlorpromazine (Largactil, Specia), carbachol (Doryl, Merck), and lipopolysaccharide from *Escherichia coli* (pyrogen).

RESULTS

Tissue reactions connected with alterations in permeability were studied in the rat paw, where oedema was produced by injection of egg white. Groups of ten rats were given one of the following preparations 30 min. before injection of the protein: isoniazid (0.05 g./kg., subcutaneously), chlorpromazine (1 mg./kg., subcutaneously) and bacterial lipopolysaccharide (0.1 or 1.0 μg./kg., intravenously). The results are shown in Figs. 1 and 2.

The oedema was increased and prolonged by isoniazid and decreased by chlorpromazine. Bacterial lipopolysaccharide in doses of 0.1 μg./kg. increased the oedema, but with larger doses the effect of the egg white was reduced. When given with isoniazid the effects of chlor-

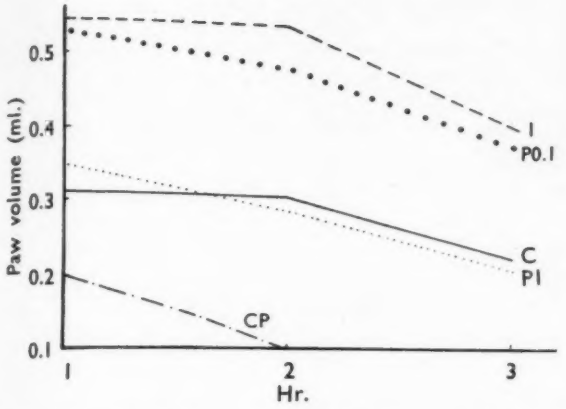

FIG. 1.—Effect of some agents on the volume of the rat paw. Each preparation was injected subcutaneously 30 min. before injection of egg white, which was used to produce oedema. **C**, control; **I**, 0.05 g./kg. isoniazid; **P1** and **P0.1**, 1 μg./kg. and 0.1 μg./kg. respectively of bacterial lipopolysaccharide (pyrogen); **CP**, 1 mg./kg. chlorpromazine. For **I**, $P=0.005$; for **P1**, $P=0.8$; for **P0.1**, $P=0.04$; for **CP**, $P<0.001$.

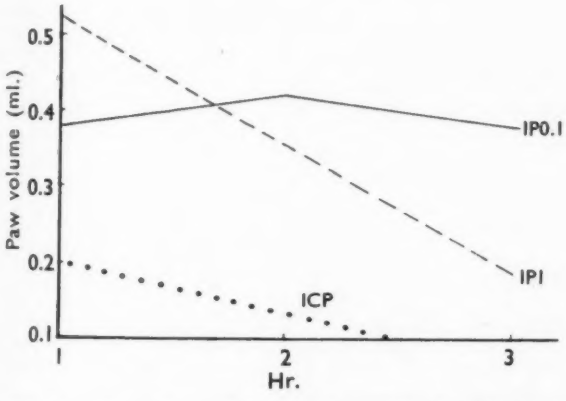

FIG. 2.—The effect of isoniazid combined with some agents on the volume of the rat paw. The combinations, which were 0.05 g./kg. isoniazid with 0.1 μg./kg. (**IP0.1**) and 1 μg./kg. (**IP1**) of bacterial lipopolysaccharide (pyrogen) and with 1 mg./kg. chlorpromazine (**ICP**), were injected subcutaneously 30 min. before injection of egg white, which was used to produce oedema. For **IP0.1**, $P=0.04$; for **IP1**, $P=0.8$; for **ICP**, $P>0.3$.

promazine and the pyrogen were similar to those obtained when they were given alone.

The effect of isoniazid on the distribution of extracellular fluid in rabbits was also studied. For this purpose, the volumes of total extracellular fluid and of plasma were measured in 10 animals before and after they had been injected with isoniazid (10 mg./kg.) twice daily for two days. The results are given in Table I, in which the coefficient I denotes the ratio of extracellular fluid to plasma.

TABLE I

DISTRIBUTION OF WATER IN CONTROL RABBITS AND IN RABBITS TREATED WITH ISONIAZID (10 mg./kg.)

I=extracellular fluid volume/plasma volume.

Control		Isoniazid	
Extracellular Volume (ml./kg.)	Plasma Volume (ml./kg.)	Extracellular Volume (ml./kg.)	Plasma Volume (ml./kg.)
252	66	307	78
I=3·8		I=3·9	

Isoniazid raised the overall retention of water in the animals without, however, modifying the rate of its distribution in tissues and blood.

Further experiments were aimed at determining the contents of isoniazid in tissues and in blood as modified by various agents. Groups of 10 rabbits were given histamine (0.5 mg./kg., subcutaneously), antazoline (5 mg./kg., subcutaneously), carbachol (15 μg./kg., subcutaneously), or chlorpromazine (1 mg./kg., subcutaneously), 60 min. before 20 mg./kg. of isoniazid, while in other groups bacterial lipopolysaccharide (0.01 μg./kg., intravenously) was given 30 min. before isoniazid and histamine was given simultaneously with isoniazid. The level of isoniazid in the blood 4, 30, 60, and 120 min. after injection is shown in Figs. 3 and 4.

All the preparations, except antazoline, significantly reduced the concentration of isoniazid in the blood. The effect of histamine was obviously dependent upon the timing of its administration. When it was given 1 hr. before isoniazid it increased the tissue permeability to such an extent that isoniazid immediately began to disappear from the blood. Even after 4 min. the concentration of isoniazid was much below that of the controls. When histamine and isoniazid were administered simultaneously the initial values were the same as for the controls. However, subsequently, the concentration of isoniazid fell rapidly and approached the values obtained in the experiments in which histamine was given before isoniazid.

For determining the isoniazid content in rat tissues, the animals were divided into groups of

ten. Group 1 served as a control. Group 2 received histamine, 0.5 mg./kg. subcutaneously 30 min. before, and 0.2 mg./kg. together with isoniazid. Group 3 received antazoline, 5 mg./kg. subcutaneously 30 min. before, and 0.5 mg./kg. intravenously together with isoniazid. Group 4 received carbachol, 15 μg./kg. subcutaneously 30 min. before, and 10 μg./kg. together with isoniazid. Group 5 was given chlorpromazine, 1 mg./kg. subcutaneously 30 min. before, and 0.25 mg./kg. intravenously at the same time as isoniazid. Group 6 was given the pyrogen, 0.1 μg./kg. intravenously 30 min. before isoniazid. The animals were sacrificed 30 min. after the injection of isoniazid. The circulatory system was rapidly perfused through the aorta with warm Ringer solution, and samples of the lung, liver and kidney were taken for isoniazid determinations. The results are shown in Figs. 3 and 4, together with curves illustrating the concentrations of isoniazid in blood.

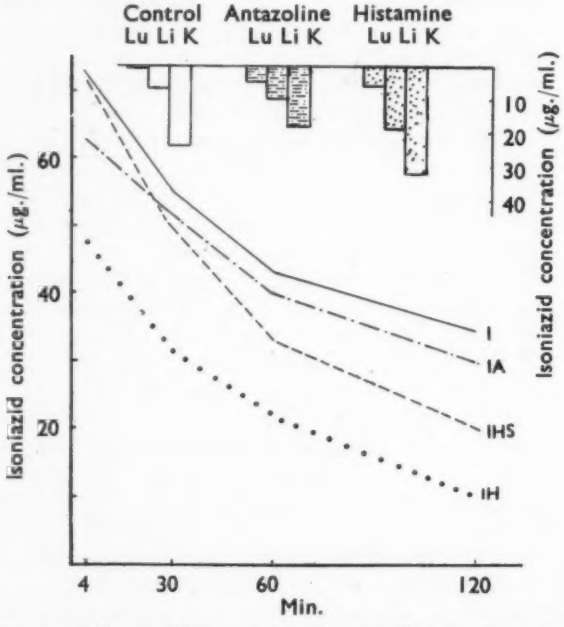

FIG. 3.—Effects of histamine and antazoline on the concentration of isoniazid in blood of rabbits and tissues of rats. The curves represent the concentration of isoniazid in the blood after injection of 20 mg./kg. isoniazid alone (I) and followed 60 min. later by 5 mg./kg. antazoline (IA), and by 0·5 mg./kg. histamine (IH). 20 mg./kg. isoniazid administered simultaneously with 0.5 mg./kg. histamine (IHS). For IA, $P=0.434$; for IHS, $P=0.015$; for IH, $P=0.001$. The columns represent the concentration of isoniazid in μg./g. in the lungs (Lu), liver (Li) and kidneys (K). For antazoline, $P=0.694$; for histamine, $P<0.001$.

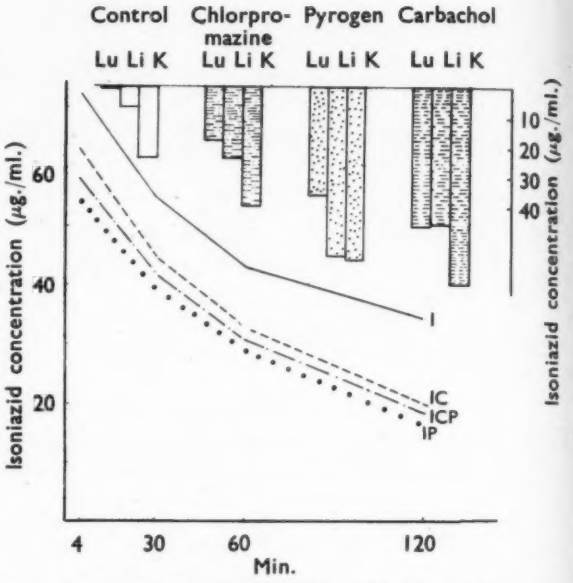

FIG. 4.—Effects of cabachol, chlorpromazine and bacterial lipopolysaccharide (pyrogen) on the concentration of isoniazid in blood of rabbits and tissues of rats. The curves represent the concentration of isoniazid in the blood after injection of 20 mg./kg. isoniazid alone (I) and followed 60 min. later by 15 μg./kg. carbachol (IC), 1 mg./kg. chlorpromazine (ICP) and by 0.01 μg./kg. bacterial lipopolysaccharide (pyrogen) (IP). For IC, $P=0.018$; for ICP, $P=0.018$; for IP, $P=0.010$. The columns represent the concentration of isoniazid in μg./g. in the lungs (Lu), liver (Li) and kidneys (K). For chlorpromazine, $P<0.001$; for bacterial lipopolysaccharide, $P<0.001$; for carbachol, $P<0.001$.

Since carbachol and the pyrogen proved very active in aiding infiltration of isoniazid into tissues, additional determinations with these two substances were carried out 1 hr. after the isoniazid injections. The results are shown in Figs. 5 and 6.

The isoniazid levels in various tissues differed according to the drugs used. Histamine, carbachol, bacterial lipopolysaccharide, and chlorpromazine significantly increased the content of isoniazid in all tissues. Consequently the reduction of isoniazid in the blood was attended by an increased infiltration into the tissues. This accounts, at least partly, for its disappearance from the blood. In spite of considerable dosage, only traces of isoniazid were found in the lungs after 30 min. when it was given alone. Even after 1 hr. the concentration was still lower than after the pyrogen. The highest concentration of isoniazid was found in the kidneys, and this is likely to be related to their excretory function.

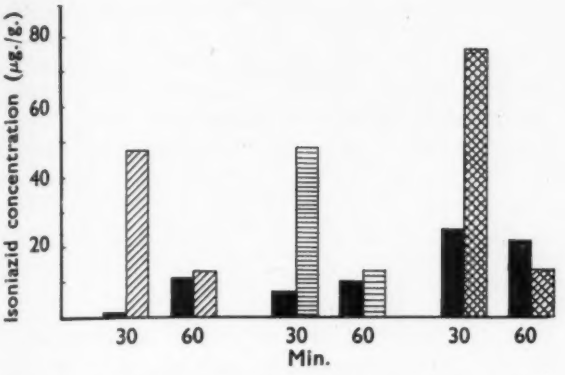

FIG. 5.—The effect of 15 μg./kg. carbachol on the concentration of isoniazid in lungs (cross-hatched), liver (horizontal hatched), and kidneys (double cross-hatched) of rats 30 and 60 min. after injection of 20 mg./kg. isoniazid. Black columns represent the concentrations after isoniazid treatment alone.

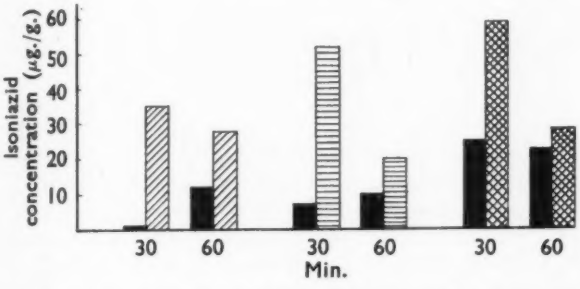

FIG. 6.—The effect of 0.01 μg./kg. bacterial lipopolysaccharide (pyrogen) on the concentration of isoniazid in lungs (cross-hatched), liver (horizontal hatched) and kidneys (double cross-hatched) of rats 30 and 60 min. after injection of 20 mg./kg. isoniazid. Black columns represent the concentrations after isoniazid treatment alone.

In the kidney the effects of the pyrogen and carbachol were most pronounced. In the control animals the concentration of isoniazid in the liver was only one-third of that in the kidneys.

When examined 1 hr. after the administration of isoniazid, the effects of carbachol and bacterial lipopolysaccharide were considerably smaller.

The overall pattern of these observations prompted us to test their possible usefulness in treating experimental tuberculosis in mice and guinea-pigs. In carrying out these experiments isoniazid was given in a dose below that currently used. It was considered that this dose was subthreshold, that is, inadequate for controlling the disease. The course of the experiments and the results obtained are shown in Tables II and III.

TABLE II

THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS IN EIGHT GROUPS, EACH OF TEN MICE, FOR 35 DAYS, WITH ISONIAZID ALONE AND IN CONJUNCTION WITH BACTERIAL LIPOPOLYSACCHARIDE, HISTAMINE OR CARBACHOL

Injections were subcutaneous. INH=isoniazid.

Group of Animals	Preparation	Dose/kg.	Mortality in Group	Average Time of Survival (Days)	Index of Tubercular Changes
I	Control	—	10/10	28·1	118
II	INH	1·0 mg.	9/10	26·9	52
III	INH+ pyrogen	1·0 mg.+ 1·0 μg.	6/10	28·4	41
IV	INH+ histamine	1·0 mg.+ 0·5 μg.	2/10	35·0	43
V	INH+ carbachol	1·0 mg.+ 20·0 μg.	4/10	27·6	31
VI	Pyrogen	1·0 μg.	10/10	18·3	72
VII	Histamine	0·5 μg.	10/10	20·5	111
VIII	Carbachol	20·0 μg.	9/10	16·4	42

TABLE III

THE TREATMENT OF EXPERIMENTAL TUBERCULOSIS IN EIGHT GROUPS, EACH OF TEN GUINEA-PIGS, FOR 62 DAYS, WITH ISONIAZID ALONE AND IN CONJUNCTION WITH BACTERIAL LIPOPOLYSACCHARIDE, HISTAMINE OR CARBACHOL

Injections were subcutaneous. INH=isoniazid.

Group of Animals	Preparation	Dose per kg.	Mortality in Group	Average Time of Survival (Days)	Index of Tubercular Changes
I	Control	—	10/10	51·1	117
II	INH	1·0 mg.	6/10	52·9	61
III	INH+ pyrogen	1·0 mg.+ 0·5 μg.	1/10	56·9	44
IV	INH+ histamine	1·0 mg.+ 0·2 μg.	2/10	56·9	41
V	INH+ carbachol	1·0 mg.+ 10·0 μg.	1/10	59·8	41
VI	Pyrogen	0·5 μg.	10/10	46·4	89
VII	Histamine	0·2 μg.	10/10	40·2	91
VIII	Carbachol	10·0 μg.	9/10	45·2	96

In evaluating the results two criteria were used: the time of survival and the index of tubercular changes. The second criterion was used only in those instances where the survival time suggested that the results were not worse than those of controls. The first criterion has only a negative value in that it is helpful to show in which groups the results were worse than the control groups. It cannot be a definitive criterion since the proper estimation of the survival time is hampered by the killing of all the animals at the moment of death of the last animal in the control group. In the first experiment, results statistically superior to those of the controls were obtained from animals treated with isoniazid alone, and with isoniazid given in conjunction with bacterial lipopolysaccharide, histamine or carbachol. Isoniazid given with carbachol proved superior to isoniazid given alone; when given in conjunction with the pyrogen or histamine it was also slightly more effective. This is of interest because the pyrogen, histamine and carbachol significantly shortened survival time when used alone.

From Table III it can be seen that in guinea-pigs the treatment with isoniazid combined with the pyrogen or histamine or carbachol gave a reduced mortality rate and a lengthening of survival time. The Tubercular Index was significantly smaller than in the control group. Bacterial lipopolysaccharide and histamine given without isoniazid did not aggravate the course of the disease as they did in the experiment with mice recorded in Table II. All groups treated by these three combinations gave better results than the group treated with isoniazid alone, though the difference was not statistically significant at the 95% level.

Discussion

Oedema of the rat's paw due to injection of egg white provided a suitable criterion for assessing the alteration in tissue permeability due to antitubercular drugs. Isoniazid increased and prolonged the oedema and in other experiments streptomycin and *p*-aminosalicylic acid had a similar effect (Jakimowska and Venulet, 1957). To some extent the results reported here are inconsistent with those of Theobald (1954), who found isoniazid to inhibit oedema production in analogous experiments with dextran. This discrepancy may be explained by a difference in the mechanism of formation of the two kinds of oedema.

Histamine, carbachol, bacterial lipopolysaccharide and chlorpromazine considerably facilitated the accumulation of isoniazid in all the tissues examined, though the results with carbachol and pyrogen in which tissue concentrations were estimated after both 60 and 30 min. indicate that these effects are of short duration. Nevertheless the concentration of antitubercular drugs in the tissues might be of therapeutic value, and this was further examined by treating infected mice and guinea-pigs with isoniazid alone and in conjunction with those drugs which increased tissue permeability. From these experiments and from others (in which a total of 360 mice and 240 guinea-pigs were used), increasing tissue permeability appeared to be a valuable complement to isoniazid treatment. The relative effectiveness of the drugs used is not yet quite certain, but carbachol appeared to give somewhat superior results to the pyrogen and histamine. However, the side-effects of carbachol and histamine are sufficiently profound to limit their value while bacterial lipopolysaccharide with its milder effects is worthy of further investigation.

Previous work on the effect of carbachol, histamine, and bacterial lipopolysaccharide on the course of tuberculosis sheds little light on the problems raised by the present work because they have been used alone and not in conjunction with an antitubercular drug. The effects of histamine are usually believed to be unfavourable and those of antihistamine drugs favourable (Garbiński, 1953; Judd and Henderson, 1949; Duca and Scudi, 1949). Mitchell (1956) points out that there is some evidence that histamine stimulates the hypophyseal-adrenal system and this alone might lead to unfavourable results, but numerous authors have found combined treatment with isoniazid and corticotrophin or cortisone to be effective (Fortier and Favez, 1952; Quiring, Perlia, and Nicole, 1953; Favez and Fortier, 1953). Our results suggest that, in addition, histamine has an important direct action on tissues and vessels. In the light of our experiments antazoline is of no value and chlorpromazine has an intermediate activity, perhaps aiding permeability by its ganglionic blocking and sympatholytic actions. Kellentei and Föllders (1954) showed that acetylcholine enhances the permeability of the blood-brain barrier and carbachol favours the accumulation of phenytoin in the brain. This may well be due to a similar mechanism (Porszasz, Venulet, and Gibiszer-Porszasz, 1954).

The role of bacterial lipopolysaccharide is most difficult to evaluate, since little work has been performed on substances of this type. Recently, Dubos and Schaedler (1956) showed the favourable effects exerted by bacterial lipopolysaccharides on the course of tuberculosis in mice.

This preparation distinctly raises the level of plasma β- and γ-globulins (Piechocki and Venulet, 1957), but the effect of pyrogens on permeability was hitherto unknown. In this connexion, it is interesting that Rowley (1956) calls attention to the positive role of pyrogens in immunity.

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THE EFFECT OF SUBSTANCE P ON THE SUPERIOR CERVICAL GANGLION OF THE CAT

BY

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Intra-arterial injections of substance P in doses from 10 to 30 units potentiated the response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve, while higher doses (30 to 100 units) usually depressed the response. The stimulating action of acetylcholine on the superior cervical ganglion (as judged by the response of the nictitating membrane) was also potentiated by substance P. The responses of the nictitating membrane to adrenaline, noradrenaline and tyramine were potentiated by substance P as well. It is concluded that the potentiating effect of substance P on the response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve is probably due to sensitization of acetylcholine receptors in the postsynaptic neurone. The present experiments do not explain how substance P potentiated the response to sympathomimetic amines.

It has been shown that substance P may restore peristalsis when injected into the lumen of the isolated guinea-pig ileum in which the peristaltic reflex has been abolished by fatigue, by external or internal application of 5-hydroxytryptamine, or by lowering the temperature of the bath (Beleslin and Varagić, 1958). Substance P, when acting on the outside of the isolated guinea-pig ileum, blocks the peristaltic reflex (Beleslin and Varagić, 1959). In previous work some evidence was obtained that this block of peristalsis might be at least partly produced by the action of substance P on the intestinal ganglia. On the other hand, Lechner and Lembeck (1958) recorded the electroencephalogram of the cortex and of the hippocampus in rabbits after an injection of 30 to 100 units of substance P into the carotid artery and found that there was a decrease in amplitude and an increase in frequency which was most pronounced in 1 to 2 min. It was therefore of interest to investigate the effect of substance P on autonomic ganglia.

METHODS

Cats of both sexes, weighing 1.5 to 3 kg., were used. After inducing anaesthesia with ether, 80 mg./kg. of chloralose was injected intravenously. Intra-arterial injections were made into the central end of the lingual artery while occluding the external carotid artery. Thus the injected substance was diverted towards the superior cervical ganglion.

The contractions of the nictitating membrane were recorded with an isotonic lever fitted with a frontal writing point magnifying the movements of the membrane ten times. The cervical sympathetic chain was divided and, when stimulated electrically, its peripheral end was placed on shielded electrodes and covered with liquid paraffin. For stimulation an electronic stimulator delivering square wave pulses was used. The pulses had a duration of 0.8 msec. and a frequency between 4 and 15 per sec.

In some experiments the intra-arterial injections were made without occluding the external carotid artery, thus allowing the injected substance to act directly on the nictitating membrane.

Substance P was extracted and purified according to the method described by Zetler (1956).

The following substances were used: substance P, acetylcholine hydrochloride, adrenaline hydrochloride, noradrenaline bitartrate and tyramine hydrochloride. With the exception of substance P all doses are expressed in terms of the salts.

RESULTS

Substance P and Sympathetic Nervous Stimulation. — The intra-arterial injection of substance P in doses from 10 to 100 units changed the response of the nictitating membrane to submaximal stimulation of the preganglionic sympathetic nerve. With the stimulation in periods of 5 sec. every minute, a series of contractions of the nictitating membrane was recorded as shown in Fig. 1. At C the external carotid artery was occluded, and an intra-arterial injection of 18 units of substance P increased the response of the nictitating membrane to the preganglionic stimulation. The external carotid artery was

opened (at O) 10 min. after the injection of substance P, thus allowing the substance to reach the nictitating membrane in a high concentration. The response to preganglionic stimulation was now reduced and 8 min. later was completely abolished. The complete block lasted 15 min. after which the response gradually returned to normal. The amount of substance P which was injected in this experiment did not itself stimulate the ganglion and caused no contraction of the nictitating membrane in the absence of preganglionic stimulation. This type of response was obtained in five out of eleven experiments.

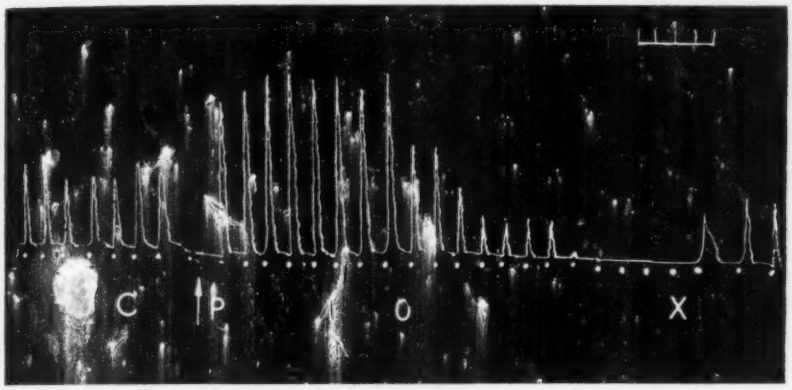

FIG. 1.—Cat, chloralose. Contractions of the nictitating membrane. At dots: submaximal preganglionic stimulation of the cervical sympathetic nerve (5 pulses/sec., 0·8 msec., 7 mA) for 5 sec. every min. At C, the external carotid artery was occluded. At P, 18 units of substance P was injected into the lingual artery. At O, the external carotid artery was opened. At X, the kymograph was stopped for 15 min. Time, 1 min.

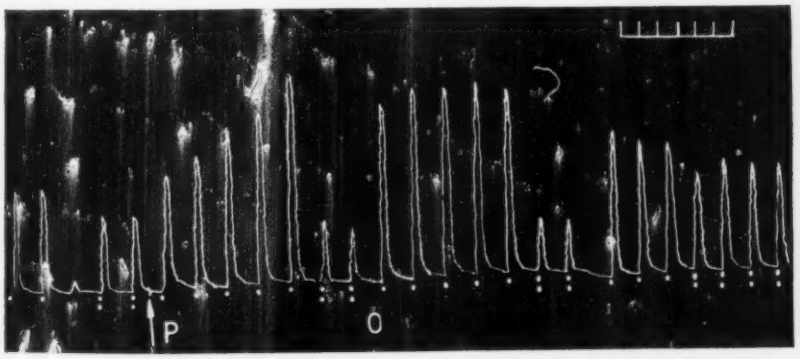

FIG. 2.—Cat, chloralose. Contractions of the nictitating membrane. The external carotid artery was occluded. At every single dot: submaximal preganglionic stimulation of the cervical sympathetic nerve (5 pulses/sec., 0·8 msec., 2 mA) for 10 sec. every 90 sec. At every two dots: submaximal postganglionic stimulation of the cervical sympathetic nerve. At O, the external carotid artery was opened. At P, 18 units of substance P was injected into the lingual artery. Time, 1 min.

The response of the nictitating membrane to stimulation of the postganglionic sympathetic nerve did not change significantly after intra-arterial injection of substance P. A typical result is shown in Fig. 2. The response of the nictitating membrane to preganglionic stimulation was potentiated immediately after injection of substance P. On the other hand, the response to postganglionic stimulation was unchanged. At O the external carotid artery was opened. After 10 min. the response to preganglionic stimulation was still potentiated but gradually declined, whereas the response to postganglionic stimulation was potentiated, as shown on the right-hand side of the figure.

In some experiments no initial increase in the response to preganglionic stimulation was observed. In these experiments the intra-arterial injection of substance P in doses from 30 to 100 units caused only a gradual decline in the response which sometimes was followed by a complete block. Such a result is shown in Fig. 3 (here the block of the response lasted for more than 45 min.). In four experiments a delayed potentiation of the effect of preganglionic sympathetic stimulation was observed. This effect was observed 30 to 120 min. after injection of substance P.

Substance P and Acetylcholine.—It has been shown previously that some substances may potentiate the response of the superior cervical ganglion to acetylcholine. Thus Konzett (1952; cited by Trendelenburg, 1956) found that histamine potentiated the response of the perfused superior cervical ganglion to acetylcholine. Trendelenburg (1956) confirmed this and found that both 5-hydroxytryptamine and pilocarpine shared this action. In the present experiments substance P also poten-

tiated the response of the nictitating membrane to acetylcholine. A typical result is shown in Fig. 4. The intra-arterial injection of 30 μg. of acetylcholine while the external carotid artery was occluded caused a contraction of the nictitating membrane due to a direct effect on the ganglion cells. These contractions of the nictitating membrane were increased after intra-arterial injection of 100 units of substance P. This effect was clear 12 min. after injection of substance P and it was regularly observed.

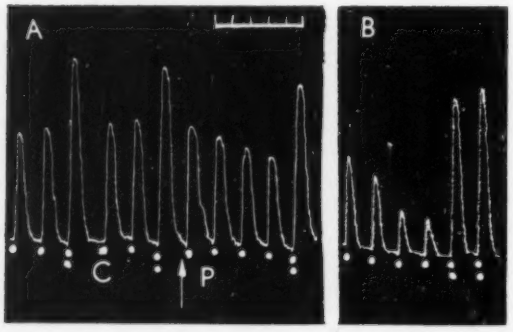

FIG. 3.—Cat, chloralose. Contractions of the nictitating membrane. At every single dot: submaximal preganglionic stimulation of the cervical sympathetic nerve (4 pulses/sec., 0·8 msec., 1·2 mA) for 10 sec. every 90 sec. At every two dots: submaximal postganglionic stimulation of the cervical sympathetic nerve (4 pulses/sec., 0·8 msec., 0·1 V.) for 10 sec. every 90 sec. At **C** the external carotid artery was occluded. At **P**, 100 units of substance P was injected into the lingual artery. **B** was taken 20 min. after the injection of substance P. Time, 1 min.

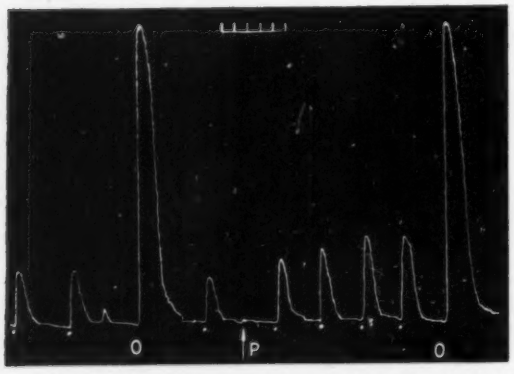

FIG. 4.—Cat, chloralose. Contractions of the nictitating membrane. At dots, 30 μg. of acetylcholine was injected into the lingual artery while the external carotid artery was occluded. At **O**, the same dose of acetylcholine was given while the external carotid artery was patent. At **P**, 100 units of substance P was injected into the lingual artery. Time, 1 min.

Substance P and Sympathomimetic Amines.—The effect of substance P on the response of the nictitating membrane to sympathomimetic amines was examined while the external carotid artery was patent, thus allowing the sympathomimetic drugs to act directly on the nictitating membrane. In the majority of the experiments it was found that the intra-arterial injection of substance P potentiated the response of the nictitating membrane to noradrenaline, adrenaline and tyramine. Fig. 5 shows such a result in which the response to noradrenaline was potentiated. This effect did not appear immediately after injection of substance P, but after an interval usually of 15 to 20 min.

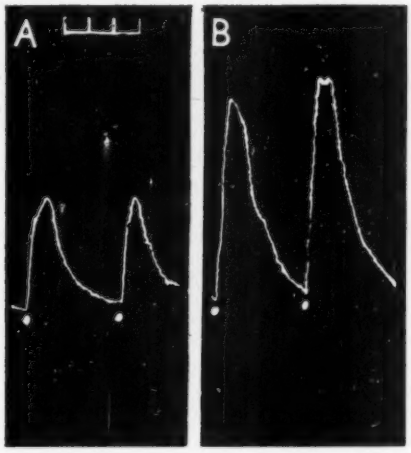

FIG. 5.—Cat, chloralose. Contractions of the nictitating membrane produced by intra-arterial injections of 20 μg. noradrenaline at the dots (the external carotid artery open). **A**, control. **B**, 20 min. after injection of 18 units of substance P into the lingual artery while the external carotid artery was open. Time, 1 min.

DISCUSSION

These experiments show that ganglionic transmission in the superior cervical ganglion of the cat may be potentiated or depressed by substance P. The potentiating effect was usually observed after small doses of substance P (10 to 30 units) and depression was usually produced by higher doses (100 units). It is known from the work of Trendelenburg (1956) that a variety of chemically unrelated substances may potentiate or depress ganglionic transmission. These substances can be divided into three groups: the nicotine-like substances (choline esters and nicotine), the sympathomimetic amines (adrenaline and noradrenaline) and a third group of drugs (including histamine, 5-hydroxytryptamine

and pilocarpine). Although all these substances potentiate sympathetic synaptic transmission, depression was obtained only with drugs of the first two groups. Substance P is known to be a polypeptide and hence chemically unrelated to any of these substances, but it can alter synaptic transmission in the sympathetic ganglion.

Substance P was also found to potentiate the response of the ganglion cells to intra-arterial injections of acetylcholine. This effect may be explained by the sensitization of acetylcholine receptors in the postsynaptic neurone. On the other hand, depression by substance P may be due to inhibition of the acetylcholine receptors or to non-specific actions of impurities in the substance P itself.

It is very difficult to explain the mechanism by which substance P potentiates the response of the nictitating membrane to the sympathomimetic amines which were used in the present experiments. The potentiating effect of substance P on the response of the nictitating membrane to postganglionic stimulation, which was occasionally seen, is in accordance with the finding that the response to noradrenaline was also potentiated.

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THE PHARMACOLOGICAL ACTIONS OF (*m*-HYDROXYPHENETHYL)TRIMETHYLAMMONIUM (LEPTODACTYLINE)

BY

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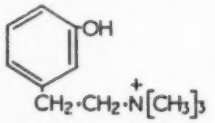
Leptodactyline is a naturally occurring quaternary ammonium base, which is found in large amounts in the skin of some South American amphibians of the genus *Leptodactylus*. It is the first example of a *m*-hydroxyphenylalkylamine in the living organism. In vertebrates it had powerful nicotinic actions and a marked neuromuscular blocking effect. This was considered, on the basis of experimental evidence, to be of the " depolarizing " type.

Leptodactyline [(*m*-hydroxyphenethyl)trimethylammonium] occurs naturally in the skin of some amphibian species of the genus *Leptodactylus*. It is the first *m*-hydroxyphenylalkylamine so far discovered in the living organism. It seems very probable that its remote precursor is *m*-tyrosine, an amino acid as yet unknown in nature.



Leptodactyline was first identified in extracts of *Leptodactylus* skin by Erspamer and Vialli (1952); its isolation and the determination of its chemical constitution were carried out by Erspamer (1959), and the proposed formula was confirmed by synthesis (Pasini, unpublished studies).

The present paper gives an account of the main pharmacological properties of leptodactyline.

METHODS

Leptodactyline Samples. — Both natural and synthetic leptodactyline picrate (molecular weight, 408.4; 1 mg. is equivalent to 0.44 mg. of the base) were used throughout the experiments. The doses given are of the picrate. The natural product was obtained as described by Erspamer (1959); the synthetic product was prepared by Dr. C. Pasini, of the Farmitalia Research Laboratories, Milan. The different samples of natural and synthetic leptodactyline showed the same chemical and paper-chromatographic characteristics and possessed exactly the same biological activity.

Pharmacological Methods.—The pharmacological methods used in the present investigation are in part identical to those described in our papers on murexine (Erspamer and Glässer, 1957, 1958).

The anaesthetics were chloralose (70 mg./kg.), pentobarbitone (30 mg./kg.), butallylonal [5-(2-bromoallyl)-5-s-butylbarbituric acid, Pernocton; 60 mg./kg.], and urethane (1 to 1.3 g./kg.), each given intravenously. Cats and dogs received intravenous injections into the femoral vein, rabbits into the marginal vein of the ear or the jugular vein, and rats and mice into the tail vein. Dogs were also given injections into the carotid artery through a plastic cannula in the thyroid artery. Injections were intravenous unless otherwise stated.

Blood pressure was measured from the femoral or carotid artery. Respiration was recorded by a tambour connected to a tracheal cannula. Adequate oxygenation of the blood during apnoea was maintained by a respiratory pump.

Neuromuscular blocking action was tested on the cat, dog, rabbit, and rat nerve-gastrocnemius preparations, prepared as described by Burn (1950). Maximal stimuli (rectangular current pulses of 7 msec. duration) were applied to the sciatic nerve through a unipolar electrode at a rate of 5 to 10 pulses/min., and the contractions were recorded semi-isometrically.

The rat phrenic nerve-diaphragm preparation, as described by Bülbring (1946), was used in a few experiments. The drug was left in contact with the tissue for 3 to 5 min.

The isolated frog rectus abdominis muscle, the leech dorsal muscle, the rabbit and guinea-pig atrium, and the guinea-pig, rabbit, rat, and cat intestine were prepared and employed in the usual manner.

Functional adrenalectomy was carried out as described by Holmstedt and Whittaker (1958), and

carotid sinus denervation by the procedure of Gollwitzer-Meier and Pinotti (1943).

Results

Pharmacological Actions

General Effects

The basic action of leptodactyline in mammals is to paralyse skeletal muscle and to stimulate ganglia powerfully. Death seems to be caused mainly by anoxia due to paralysis of the respiratory muscles; it cannot, however, be excluded that the consequences of ganglionic stimulation may contribute to death.

Muscular paralysis and respiratory depression were generally preceded or accompanied by short-lived polypnoea and by muscular twitches and fasciculations all over the body, salivary hypersecretion, lachrymation, mydriasis alternated with myosis, intestinal borborygmi, defaecation and micturition. When death occurred muscular twitchings and fasciculations persisted for several minutes after death.

The LD50 of leptodactyline picrate in mice was 3.3 mg./kg. by intravenous route, and approximately 325 mg./kg. by mouth; the dose causing head drop in rabbits (ED50) was approximately 0.2 mg./kg., intravenously.

In birds leptodactyline, like all depolarizing muscle relaxants, provoked contracture (extension cramp of the legs and opisthotonos) instead of muscular paralysis. Myosis alternated with mydriasis and evacuation of the bowels was sometimes observed at the same time. In pigeons the minimum active intravenous dose was 20 to 25 μg./kg. and the LD50 120 to 180 μg./kg. Frogs and fishes were paralysed, like mammals. The paralysing dose (ED50) of leptodactyline in frogs, following injection into the dorsal lymphatic sac, was approximately 0.5 mg./kg. The righting reflex disappeared after 5 to 10 min., and returned to normal after 20 to 35 min.

Neuromuscular Block

Sciatic Nerve-Gastrocnemius Muscle Preparation.—A comparison of the paralysing action of different doses of leptodactyline on cat, dog, rabbit, and rat gastrocnemius is given in Table I. Neuromuscular transmission was less sensitive to leptodactyline in the dog than in the cat, while that in the rabbit and in the rat was still less sensitive. In this respect leptodactyline behaved like murexine.

As with all other blocking agents, the repeated administration of leptodactyline at brief intervals produced a cumulative effect. As with suxamethonium and murexine, the twitch reduction was sometimes preceded by a short-lived increase in the tension of the muscle, as shown by the potentiation of some twitches, and, between the contractions, fasciculations of the muscle could be seen. Tubocurarine could antagonize the muscle-relaxant action of leptodactyline. Intravenous injection of 100 μg./kg. of the drug, given during

TABLE I

THE PARALYSING ACTION OF LEPTODACTYLINE PICRATE ON THE SCIATIC NERVE-GASTROCNEMIUS MUSCLE PREPARATION OF DIFFERENT SPECIES

Twitch reductions are the maximum changes expressed as the % of control twitch. Recovery times refer to 50% recovery. — means no experiments. 0 means no depression of twitch.

Dose of Leptodactyline (μg./kg.)	Cat		Dog		Rabbit		Rat	
	Twitch Reduction (%)	Recovery Time (min.)	Twitch Reduction (%)	Recovery Time (min.)	Twitch Reduction (%)	Recovery Time (min.)	Twitch Reduction (%)	Recovery Time (min.)
20	2–15	—	—	—	—	—	—	—
40	10–15	½–2	—	—	—	—	—	—
50	35	2	20	2	0	—	—	—
80	60–90	2	—	—	0	—	0	—
100	85–90	3	70–80	2½	0	—	0–5	—
150	90–95	3	—	—	0	—	—	—
200	95–99	4–6	—	—	5–10	1	10	2–3
400	100	6	95	5	15–45	2	25	5
1,000	100	20	95–98	12	75–99	4–5	40	8–9
1,500	100	50	—	—	—	—	—	—
2,000	100	60	—	—	—	—	72	21

an intravenous infusion of 45 μg./kg./min. leptodactyline which depressed the twitch by 75%, promptly abolished muscular paralysis. The intravenous injection of 5 mg./kg. of choline chloride during the recovery from muscle relaxation produced by 300 to 500 μg./kg. of leptodactyline caused a transient increase of the depressed muscular twitch.

Rat Diaphragm.—Leptodactyline (7 μg./ml.) reduced by 15% the amplitude of muscular twitches. With 15 μg. and 30 μg./ml. the reduction was 30 and 80% respectively. It may

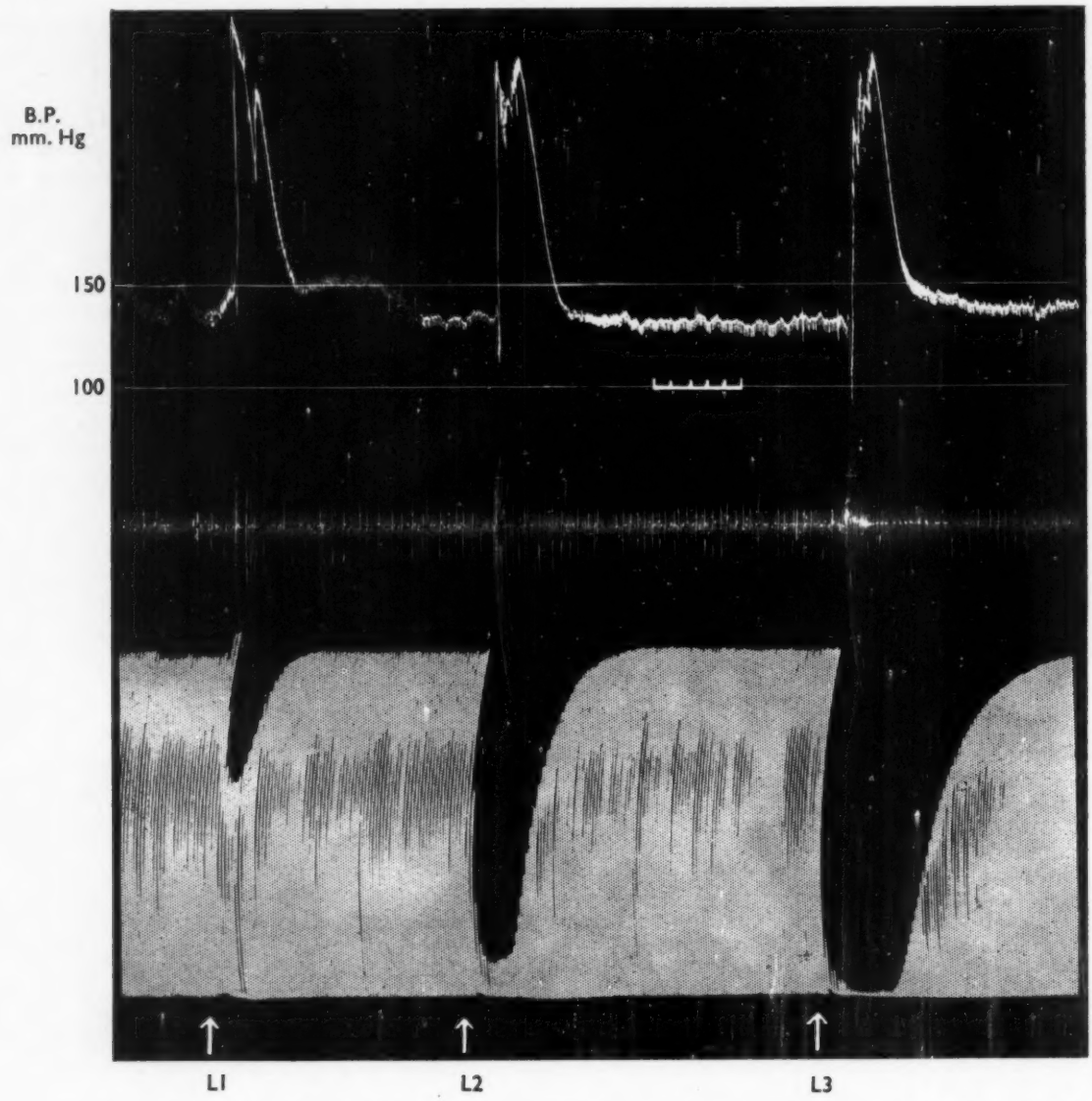


FIG. 1.—Cat anaesthetized with chloralose. Upper tracing, blood pressure. Middle tracing, respiration. Lower tracing, twitches of the gastrocnemius muscle in response to single maximal stimuli to the sciatic nerve. Time, 1 min. Leptodactyline picrate produced a sharp rise in blood pressure, a short-lived stimulation of respiration and a neuromuscular block. The pressure response was maximal with all the three doses of leptodactyline tested (50, 100 and 200 μg./kg., at **L1, L2** and **L3**), respiratory stimulation and neuromuscular block increased with the dose. With 200 μg./kg. there was no impairment of respiratory movements, in spite of a nearly complete neuromuscular block of the gastrocnemius.

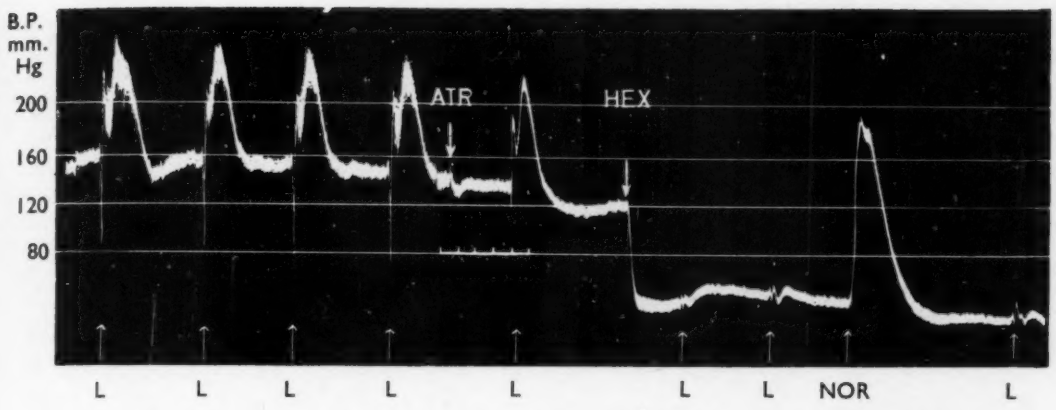


FIG. 2.—Blood pressure of a dog anaesthetized with pentobarbitone. At **L**, 30 μg./kg. leptodactyline picrate; at **ATR**, 0.5 mg./kg. atropine sulphate; at **HEX**, 4 mg./kg. hexamethonium bromide; at **NOR**, 1.5 μg./kg. noradrenaline. All the injections were given intravenously. Time, 1 min. The pressure response to leptodactyline showed no tachyphylaxis. The short-lived primary pressure fall was abolished by atropine, and the pressure rise by hexamethonium.

be seen that this preparation was not very sensitive to leptodactyline.

Blood Pressure

Cat.—In cats anaesthetized with chloralose, rapid intravenous injections of leptodactyline caused a very transient hypotension (10 to 70 mm. Hg, according to the dose), followed by a more sustained rise of blood pressure (Fig. 1). With high doses of leptodactyline (100 μg./kg. and more) the blood pressure rose by 80 to 130 mm. Hg, but the sharp pressure rise was generally followed by a secondary fall lasting 10 to 20 min. The pressure changes elicited by low doses of leptodactyline were not accompanied by changes in the heart rate; moderate or high doses, on the contrary, produced bradycardia, eventually followed by a period of tachycardia. The minimum dose of leptodactyline active on the blood pressure of the intact cat was 1 to 2 μg./kg.

In contrast to the intact animal, the spinal cat nearly always showed a simple rise of blood pressure after intravenous leptodactyline. Very often the rise was proportional to the dose administered. Only in some cats was hypertension preceded by a weak, fleeting fall of blood pressure.

Atropine sulphate (1 mg./kg.) abolished the initial fall of blood pressure; hexamethonium bromide (2 to 5 mg./kg.) and diethylaminomethyl-benzo-1,4-dioxan (Prosympal, 1 to 5 mg./kg.) reduced or abolished the leptodactyline hypertension. In the spinal cat, the rise of blood pressure produced by 25 to 50 μg./kg. of leptodactyline was reduced by about 25% following functional adrenalectomy. This may signify that only 25% of leptodactyline hypertension is due to release of medullary catecholamines.

Dog.—In dogs anaesthetized with chloralose, pentobarbitone, or butallylonal the usual response to intravenous leptodactyline was an abrupt fall of blood pressure lasting a few seconds, followed by a sharp, more sustained rise (Fig. 2). However, with very low doses of leptodactyline, hypotension, with no change in the heart rate, was sometimes the only response. With moderate doses of leptodactyline (20 to 50 μg./kg.) hypotension of 40 to 60 mm. Hg was followed by hypertension of 40 to 100 mm. Hg. The heart rate was at first reduced, then increased.

Often hypertension was roughly proportional in intensity and duration to the dose of leptodactyline, and repeated doses of the same magnitude elicited similar responses.

As in the cat, the short-lived primary hypotension was antagonized by atropine (0.5 mg./kg.) and the rise of blood pressure by hexamethonium (3 mg./kg.) (Fig. 2). Prosympal reduced the hypertension, leaving the primary hypotension completely unaffected. Bilateral vagotomy and denervation of the carotid sinuses caused very little, if any, effect either on the initial fall or the subsequent rise of blood pressure due to leptodactyline. Intracarotid injections of leptodactyline produced approximately the same initial hypotension as intravenous injections, but were several times less effective in causing the rise of blood pressure, which was also somewhat delayed (Fig. 3).

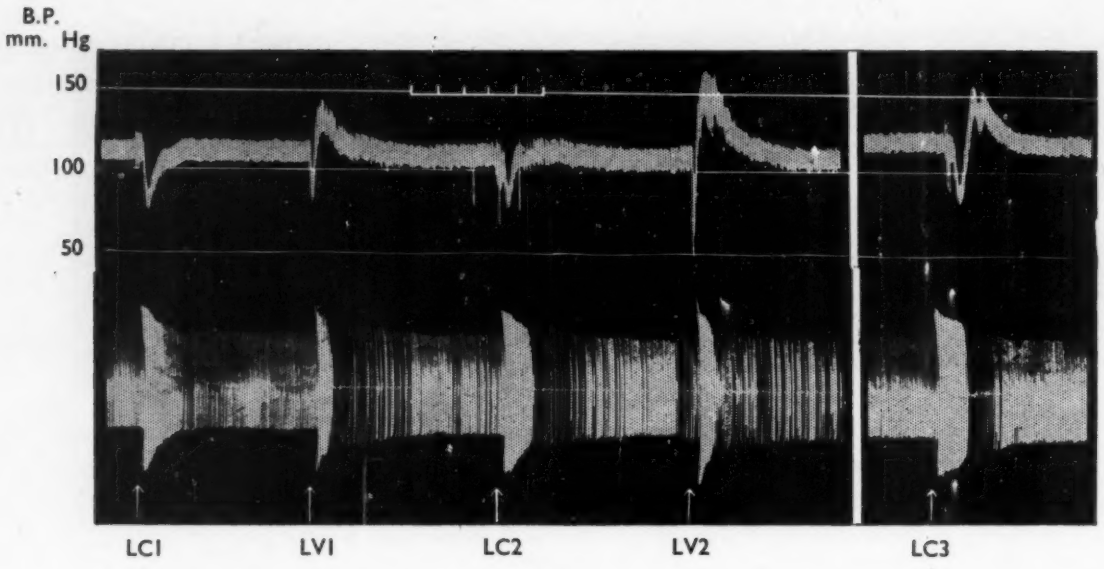


FIG. 3.—Dog anaesthetized with butallylonal. Upper tracing, blood pressure. Lower tracing, respiration. At **LC1, LC2** and **LC3**, 30, 60 and 150 μg./kg. leptodactyline picrate into the carotid artery; at **LV1** and **LV2**, 30 and 60 μg./kg. leptodactyline intravenously. Time, 1 min. The primary hypotensive phase produced by leptodactyline remained apparently unchanged following injection into the carotid artery; the hypertensive phase, on the contrary, was lacking or reduced in intensity and delayed in time. Respiratory stimulation was more intense and prolonged following injection of leptodactyline into the carotid artery.

It appears probable that the initial hypotension is essentially due to a cardiac action of leptodactyline and that the hypertension is due both to ganglionic stimulation and to release of pressor amines from the body stores. Reflex or direct stimulation of the vasomotor centres in the medulla does not play any important role.

Rabbit.—In rabbits anaesthetized with urethane leptodactyline nearly always provoked a more or less pronounced hypotension (20 to 70 mm. Hg). This was transient with small doses (2 to 20 μg./kg.), and more persistent with high doses (50 to 500 μg./kg.). The first fall of blood pressure was often followed, after return to the starting level, by a secondary, long-lasting fall. Rarely a slight, short-lived rise of blood pressure was interposed between the two hypotensive phases. Hexamethonium (5 mg./kg.) had no effect on the hypotension after leptodactyline.

Heart

Guinea-pig Atrium. — This preparation was considerably more sensitive to leptodactyline than the rabbit atrium. The predominant effect was a reduction in the amplitude of beat: 4 to 10 min. following 0.5 μg./ml. the reduction was 25 to 60%, and after 2 μg./ml. it was 55 to 95%. The frequency was similarly reduced, by 9 and 50%, respectively, at the above concentrations. A concentration of 1 μg./ml. of leptodactyline was equipotent to 0.1 μg./ml. acetylcholine bromide.

Following 0.1 μg./ml. atropine sulphate weak concentrations of leptodactyline were ineffective; high concentrations (10 μg./ml.) caused a moderate increase in the frequency (15 to 30%) and a more conspicuous increase in the amplitude (40 to 100%) of beat. This effect was similar to that produced by 1 to 3 μg./ml. nicotine tartrate. Very high concentrations of hexamethonium (50 to 300 μg./ml.) were required to abolish the depressive effects of leptodactyline on the normal atrium; much smaller concentrations (0.1–0.2 μg./ml.), on the contrary, could reduce or abolish the stimulant effects of leptodactyline on the atropinized atrium (Fig. 4).

Rabbit Atrium.—The isolated rabbit atrium responded to leptodactyline (1 to 50 μg./ml.) with a prolonged reduction in the frequency of contractions, which often persisted for some time after washing with fresh nutrient solution. The amplitude of beat was moderately reduced with weak concentrations (1 to 5 μg./ml.) of

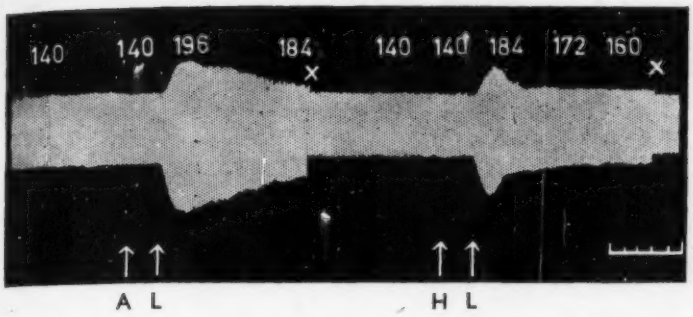


FIG. 4.—Guinea-pig atrium, suspended in oxygenated Ringer-Locke solution at 30°. At **L**, 10 μg./ml. leptodactyline picrate; at **A**, 0.1 μg./ml. atropine sulphate; at **H**, 0.01 μg./ml. hexamethonium bromide + 0.1 μg./ml. atropine sulphate; at **X**, washing. Time, 1 min. After atropine, leptodactyline produced an increase in amplitude and frequency of beats. This effect was considerably reduced by low doses of hexamethonium.

leptodactyline. With higher concentrations the initial negative inotropic effect turned into a positive effect within a few minutes. After 5 to 15 min. the amplitude of beat was sometimes increased, sometimes decreased.

Following atropine sulphate (1 μg./ml.), leptodactyline was completely inactive in concentrations of 1 to 3 μg./ml. In concentrations of 5 μg./ml., on the contrary, it regularly caused a marked increase both in the frequency and the amplitude of beat. The first effect was persistent, the second often turned into a decrease in the amplitude of beat.

High concentrations of hexamethonium tartrate (100 to 200 μg./ml.) produced a complete block of the leptodactyline effects, with the exception of a slight, transient increase in the frequency of contractions. Similarly, all the effects of leptodactyline were opposed by the simultaneous administration of atropine and Prosympal (20 μg./ml.). The stimulant effect of 15 to 20 μg./ml. leptodactyline on the atropinized rabbit atrium was quantitatively similar to that after 10 μg./ml. nicotine tartrate.

It seems highly probable that the negative effects of leptodactyline on the isolated atria are due to a stimulation of parasympathetic ganglia, and the positive effects to stimulation of sympathetic ganglia, or, more likely, to a release of catecholamines from stores within the heart. In fact, Glässer (unpublished observations) has found that stimulation by leptodactyline is reduced or abolished in atria taken from animals treated with reserpine, exactly as it is for stimulation by nicotine (Burn and Rand, 1958).

Respiration

Cat.—In cats anaesthetized with chloralose the minimum intravenous dose of leptodactyline

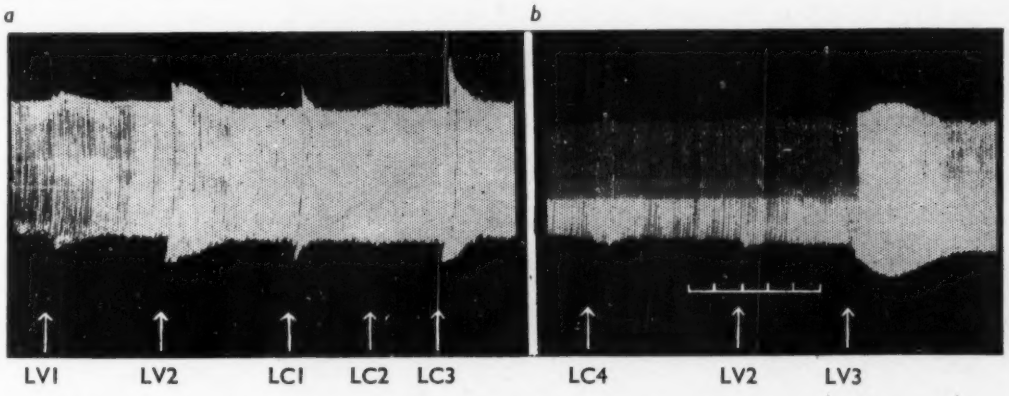


FIG. 5.—Respiration of a dog anaesthetized with butallylonal. At **LV1**, **LV2** and **LV3**, 7, 15 and 50 μg./kg. leptodactyline picrate intravenously; at **LC1**, **LC2**, **LC3** and **LC4**, 0.05, 0.5, 1.5 and 2.5 μg./kg. leptodactyline into the carotid artery. Between (*a*) and (*b*) section of both vagi and denervation of the carotid sinus areas. Time, 1 min. Leptodactyline was ten to fifteen times more active on respiration when given into the carotid artery than when given intravenously; denervation of the carotid sinus areas abolished the prompt respiratory stimulation produced by leptodactyline. With high doses of the substance a more sustained respiratory stimulation could be observed.

which affected respiration was 10 to 20 μg./kg. The usual response was a short-lived respiratory stimulation, followed by a period of slight depression (Fig. 1). High doses of leptodactyline (500 μg./kg. and more) regularly provoked respiratory arrest due to paralysis of the skeletal musculature.

Dog.—The minimum dose of leptodactyline active on respiration was 1 to 5 μg./kg. There was a very short-lived intense stimulation of respiratory movements, followed by depression or short-lasting apnoea.

Injections of leptodactyline into the carotid artery were ten to fifteen times more active than intravenous injections (Fig. 5); on the other hand, denervation of the carotid sinus areas completely abolished the respiratory response. It is therefore concluded that the above effect of leptodactyline on respiration is brought about by stimulation of the chemoreceptors of the carotid bodies. The secondary respiratory depression was probably a consequence of hyperventilation.

Following denervation of the carotid sinus areas, leptodactyline sometimes produced a different respiratory response; a stimulation which was slower in onset and more prolonged than that previously described.

Rabbit.—The usual response to leptodactyline (10 μg./kg. and more) in this species was a short-lasting apnoea followed by a brief period of respiratory stimulation.

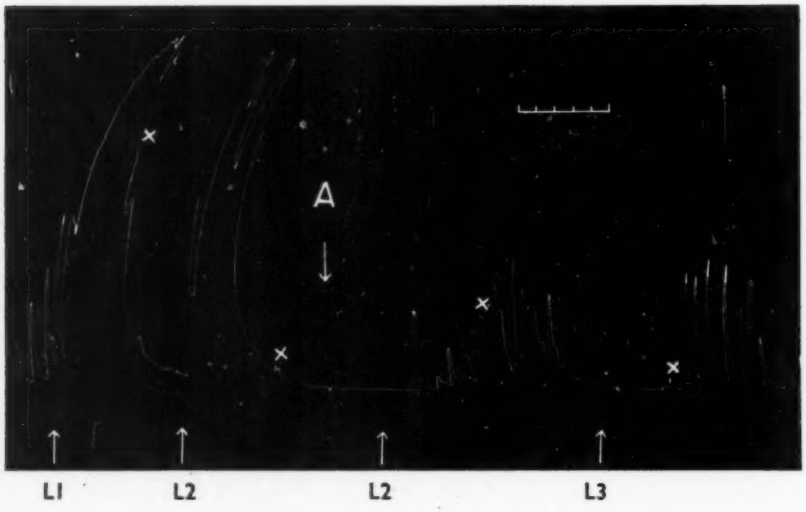


FIG. 6.—Isolated rabbit colon suspended in Tyrode solution at 37°. At **L1**, **L2**, and **L3**, 3, 10 and 30 μg./ml. leptodactyline picrate; at **A**, atropine sulphate, 1 μg./ml.; at **X**, washing. Time, 1 min. Atropine abolished the stimulant effect of leptodactyline on the rabbit colon. Tachyphylaxis was evident before atropinization.

Smooth Muscle

Intestine. — The minimum concentration of leptodactyline which caused contraction of the guinea-pig small and large intestine and the frog stomach was approximately 0.5 to 1 μg./ml. There was no good dose/response relationship and often tachyphylaxis could be observed. The rabbit large intestine showed the same sensitivity to leptodactyline. Atropine (1 μg./ml.) completely abolished the spasmogenic effect of the substance, which then produced a transient decrease in tone and reduction or suppression of spontaneous movements (Fig. 6).

The cat colon responded to leptodactyline with a contraction which was often satisfactorily proportional to the dose. Atropine (1 μg./ml.) abolished this effect; hexamethonium tartrate (0.2 to 1 mg./ml.) and mecamylamine hydrochloride (5 to 25 μg./ml.) consistently reduced it.

The results obtained on the intestinal smooth muscle, which are very similar to those produced by nicotine, are to be interpreted as the expression of a stimulation of intramural ganglia.

Other Tissues

Frog Rectus Abdominis. — Leptodactyline induced, in very low concentrations (minimum active concentration 0.01 to 0.03 μg./ml.), a contracture in the frog rectus. There was a very satisfactory dose/response relationship. Therefore, the frog rectus may be considered a very convenient and suitable preparation for the quantitative estimation of leptodactyline. The leptodactyline contracture was very similar to that produced by the methonium compounds and murexine, but differed from that provoked by acetylcholine, which had a more rapid onset and reached a plateau much more readily (Fig. 7). The relative potencies in stimulating the frog rectus following a 3 min. contact were: leptodactyline picrate, 100; murexine dipicrate, 6 to 8; acetylcholine bromide, 20 to 25; nicotine tartrate, 1 to 2.

Eserine salicylate (0.01 mg./ml.) did not potentiate the response to leptodactyline. Tubocurarine showed

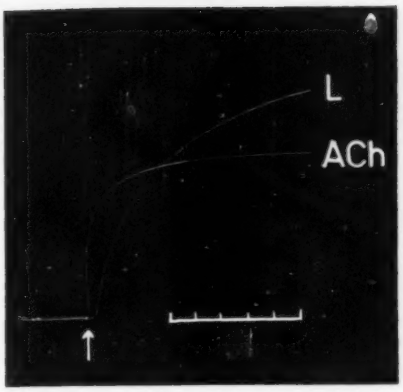


FIG. 7.—Frog rectus abdominis preparation. At arrow 0.2 μg./ml. leptodactyline picrate (**L**) and then, after washing and relaxation of the muscle, 1 μg./ml. acetylcholine bromide (**ACh**). Time, 1 min. The response caused by leptodactyline had a slower onset compared with that caused by acetylcholine and did not reach a plateau.

a strong antagonistic action: after 0.125 and 1.25 μg./ml. the contracture caused by 0.05 μg./ml. leptodactyline was reduced by 50 and 85% of the control, respectively.

Leech Muscle. — Leptodactyline caused a contracture of this preparation which closely resembled that produced by murexine (Erspamer and Glässer, 1957). The minimum active concentration was 0.5 to 1 μg./ml. There was, in several instances, an excellent dose/response relationship (Fig. 8).

Fate of Leptodactyline in the Organism

Three groups of four rats, each weighing 800 to 950 g., were given 10 mg. leptodactyline per rat, one group orally and two groups subcutaneously. Urine was collected first for a 6 hr. period, and then for a second period of 18 hr. The activity of the urine on the frog rectus abdominis was determined, and expressed in terms of leptodactyline; moreover, concentrated urine was submitted to paper chromatography.

The urinary activities, expressed in absolute values of leptodactyline and in percentages of the drug administered, were as follows:

Oral	0– 6 hr. period	42 μg.	(0·42%)
	7–24 ,, ,,	96 ,,	(0·96%)
	Total	138 ,,	(1·38%)
Subcutaneous	0– 6 ,, ,,	2,900 ,,	(29·0%)
	7–24 ,, ,,	400 ,,	(4·0%)
	Total	3,300 ,,	(33·0%)
Subcutaneous	0– 6 ,, ,,	1,300 ,,	(13·0%)
	7–24 ,, ,,	180 ,,	(1·8%)
	Total	1,480 ,,	(14·8%)

It may be seen that leptodactyline was largely destroyed in the organism, even when administered by parenteral route. Since monoamine oxidase does not seem able to attack quaternary ammonium bases (Blaschko, 1952) it is probable that other enzyme systems are involved in the inactivation of leptodactyline.

Paper chromatograms of concentrated urine demonstrated the presence of unaltered leptodactyline and of two unidentified metabolites of the substance, still bearing a free hydroxyl group in the *meta*-position. The R_F values on Whatman No. 1 paper, using the ascending technique and diazotized sulphanilic acid as a spraying reagent, were: (*a*) for the butanol + acetic acid + water (4:1:5) solvent system, leptodactyline 0.58, metabolite I 0.68, metabolite II 0.90; and (*b*) for the butanol + 25% aqueous methylamine (8:3) solvent system, leptodactyline 0.15, metabolite II 0.28.

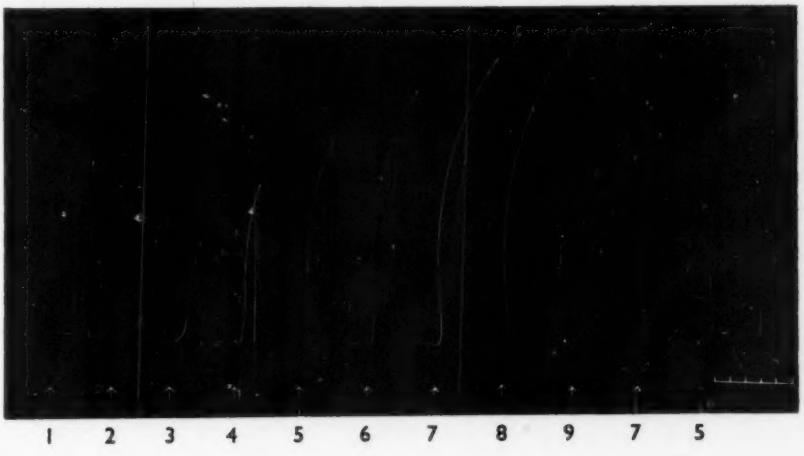


FIG. 8.—Leech muscle preparation. Contractions produced by increasing concentrations of leptodactyline picrate (in μg./ml.). Time, 1 min. There was a good dose response relationship.

Discussion

Leptodactyline is the first *m*-hydroxyphenylalkylamine to be found in the living organism, specifically in the skin of a vertebrate. It is highly probable that *m*-tyrosine represents the parent amino acid of leptodactyline. Blaschko (1950, 1958) and Mitoma, Posner, Bogdanski, and Udenfriend (1957) have shown that *m*-tyrosine, in sharp contrast to the widely distributed *p*-tyrosine, is a substrate, both *in vitro* and *in vivo*, for mammalian decarboxylase. The resultant *m*-tyramine might easily be transformed into its *N*-methylated derivatives by transmethylating enzymes which seem to be particularly active and abundant in the skin of amphibians, as shown by the occurrence in this skin of a whole series of *N*-methylated derivatives of 5-hydroxytryptamine. Bufotenidine is the trimethylammonium derivative of 5-hydroxytryptamine, as leptodactyline is the trimethylammonium derivative of the hitherto unknown *m*-tyramine.

With the description of leptodactyline and of *o*-tyrosine (Dennell, 1956), all the three isomers of hydroxyphenylalanine (that is, the three tyrosines) may be considered naturally occurring amino acids, capable of giving origin to highly active derivatives. As one of them, leptodactyline possesses the pharmacological properties shown by other natural or synthetic *N*-methylated hydroxyphenylalkylamines (such as hordenine, candicine, and coryneine). In fact, leptodactyline causes both a powerful nicotinic stimulation at autonomic ganglia and the neuromuscular junction, and a considerable neuromuscular block. Muscarinic effects seem to be lacking.

All the available experimental evidence suggests that leptodactyline should be listed among the blocking agents acting by depolarization: the neuromuscular block produced by the substance is preceded and followed in mammals by muscular fasciculations, and it is antagonized by tubocurarine; the frog rectus abdominis is contracted by leptodactyline, and so is the skeletal musculature of the intact bird.

The relation between the chemical structure and the pharmacological actions of compounds like leptodactyline [trimethyl(phenylalkyl)ammonium derivatives] will be discussed in greater detail elsewhere. Here we wish only to emphasize the fact that the presence on the benzene ring of a free hydroxyl group in the *meta*-position is of decisive importance for the intensity of pharmacological effects. Ganglionic stimulation and neuromuscular block are ten to twenty times greater for leptodactyline than for (*p*-hydroxyphenethyl)trimethylammonium (candicine) and (*o*-hydroxyphenethyl)trimethylammonium. Something similar has been observed in regard to the sympathomimetic pressor activity of the three isomers of (±)-1-(hydroxyphenyl)-2-methylaminoethanol: the *meta*-isomer (synephrine) is six and eighteen times more hypertensive than the *para*- and *ortho*-isomers, respectively (Bovet and Bovet-Nitti, 1948).

The physiological significance of the occurrence of leptodactyline in *Leptodactylus* skin is obscure, as is that of the hydroxyindolyl alkylamines and catecholamines in the skin of other amphibian species. It is rather doubtful whether it can serve for defence, since leptodactyline, at least pure leptodactyline, is almost completely inactivated, and in consequence largely ineffective when administered by oral route.

It seems possible that, in common with other amphibian extracts (Vellard, 1959), extracts of *Leptodactylus* skin have also been used by the natives of South America in the preparation of some "curares."

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RECIPROCAL POTENTIATING ACTION OF DEPOLARIZING DRUGS ON THE ISOLATED FROG RECTUS ABDOMINIS MUSCLE

BY

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Subthreshold concentrations of the depolarizing agents, acetylcholine and decamethonium, potentiated isotonic contractures of the isolated frog rectus abdominis muscle elicited by effective doses of either of these drugs or by choline in the absence of the anticholinesterases, sarin, or dyflos. Comparable potentiation of the contracture produced by decamethonium or choline could be obtained by alternate stimulation with acetylcholine in the absence of dyflos or sarin. Furthermore, dyflos alone did not effect potentiation in the absence of alternate stimulation with acetylcholine. Finally, the inhibitory action of tubocurarine, as measured by depression of the contracture elicited by decamethonium and acetylcholine, was not antagonized by sarin. It was concluded that the enhancement of the response to acetylcholine produced by dyflos and by sarin was brought about exclusively by their action as inhibitors of cholinesterase. The ability of dyflos and of sarin to potentiate contractures elicited by decamethonium or by choline may be attributed to a facilitation of the action of the depolarizing compounds by subthreshold concentrations of acetylcholine available in the presence of these anticholinesterases.

Cohen and Posthumus (1955) observed that dyflos (di-isopropyl phosphorofluoridate) and sarin (isopropyl methylphosphonofluoridate) potentiate the effects of decamethonium and choline on the isolated frog rectus abdominis muscle. Because decamethonium and choline are not esters, and are therefore not hydrolysed by cholinesterase, they concluded that dyflos, and other anticholinesterases, promote a "sensitization" of acetylcholine receptors independent of any inhibition of cholinesterase activity. They further inferred that direct, non-enzymatic sensitization "is at least partly responsible for the enhanced response of the muscle toward acetylcholine after the administration of anticholinesterases." In view of the fundamental nature of their conclusions concerning the mechanism of action of anticholinesterases, a similar study on the frog rectus has been conducted in our laboratory.

METHODS

Frog Ringer Solution. — Composition: NaCl, 0.56%; KCl, 0.0075%; $CaCl_2$, 0.01%; dextrose 0.2%. The solution was buffered with 0.02 M-tris-(hydroxymethyl-amino)methane (Tris buffer) at *p*H 7.2 and was bubbled with oxygen.

Depolarizing Drugs. — Acetylcholine chloride, choline chloride, and decamethonium bromide were made up in frog Ringer solution. The test solutions of acetylcholine were freshly prepared before each experiment.

Anticholinesterase Drugs. — Dyflos (di-isopropyl phosphorofluoridate, DFP) [Merck Ampoules]. Sarin (isopropyl methylphosphonofluoridate), obtained from Chemical Research Division of Chemical Warfare Laboratories; purity was 95% or better according to an analysis of acidity. All solutions of anticholinesterases were made up freshly before use in frog Ringer solution.

Measurement of Isotonic Contracture. — The method of preparation of the isolated muscle, addition of reagents and measurement of muscle contracture have been previously described (Fleisher, Howard, and Corrigan, 1958). Responses of the muscle to any dose of a stimulating drug were repeated until successive measurements yielded an identical rate of contracture (± 1 mm./min.) before the addition of a second depolarizing substance (for example, see Table I).

Results

Effect of Subthreshold Concentrations of Depolarizing Agents upon the Contracture Produced by Addition of a Second Depolarizing Agent

A reproducible rate of contracture of the muscle was obtained to a particular dose of decamethonium to serve as a control for the response elicited by the same dose of decamethonium in the presence of a subthreshold concentration of acetylcholine. Control measurements showed no contraction following addition of subthreshold concentrations of acetylcholine, decamethonium and choline which were approximately half of the minimal effective dose.

TABLE I

POTENTIATING EFFECT OF SUBTHRESHOLD CONCENTRATIONS OF DEPOLARIZING SUBSTANCES ON THE CONTRACTURE PRODUCED BY THE ADDITION OF A SECOND DEPOLARIZING AGENT ON ISOLATED FROG RECTUS ABDOMINIS MUSCLE

Expt. No.	Dose (μg./ml.)	Contracture (mm./min.)
1	50 μg. decamethonium	20
	50 ,, ,, ...	20
	0·05 μg. acetylcholine for 1·5 min.	No contracture
	Followed by 50 μg. decamethonium	27
	0·05 μg. acetylcholine for 1.5 min.	No. contracture
	Followed by 20 μg. decamethonium	20
2	1 μg. acetylcholine	18·0
	1 ,, ,, ...	18·5
	6 μg. decamethonium for 1·5 min.	No contracture
	Followed by 1 μg. acetylcholine ..	28·5
3	1,000 μg. choline	21
	1,000 ,, ,, ...	21
	0·05 μg. acetylcholine for 1·5 min.	No contracture
	Followed by 1,000 μg. of choline	26·5
	0·05 μg. acetylcholine for 1·5 min.	No contracture
	Followed by 800 μg. of choline ..	21
4	1 μg. acetylcholine	20·5
	1 ,, ,, ...	20
	70 μg. choline for 1·5 min. ..	No contracture
	Followed by 1 μg. acetylcholine ..	23

Results shown in Table I indicate that an increased response to decamethonium may be produced by as little as 0.05 μg./ml. of acetylcholine (Expt. No. 1). Similarly the responses to acetylcholine and to choline were potentiated by the presence of subthreshold concentrations of decamethonium and of acetylcholine (Expt. Nos. 2 and 3).

There was little or no potentiation to acetylcholine in the presence of a subthreshold concentration of choline (Expt. No. 4).

Effect of Sarin on the Potentiation of Acetylcholine Produced by Decamethonium.—A constant initial response was obtained to acetylcholine and then in the presence of a subthreshold amount of decamethonium. After washing, the response to further addition of acetylcholine was elicited twice in the absence of decamethonium at the usual interval of 1 min. Table II shows that the enhancement of response disappears rapidly after the decamethonium is washed out.

Incubation with sarin 5×10^{-7} M for 1.5 hr., and testing in the presence of 10^{-7} M sarin, resulted in a ten-fold sensitization of the muscle to acetylcholine, without its response to decamethonium

TABLE II

EFFECT OF SARIN ON THE RESPONSE PRODUCED BY THE ADDITION OF ACETYLCHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE IN THE PRESENCE OF A SUBTHRESHOLD CONCENTRATION OF DECAMETHONIUM

In B the muscle was incubated with sarin, 5×10^{-7}M for 1.5 hr. followed by measurement of responses in 10^{-7}M-sarin.

	Dose (μg./ml.)	Contracture (mm./min.)
A.	Before sarin	
	1 μg. acetylcholine	22
	1 ,, ,, ...	23
	2 μg. of decamethonium for 1·5 min.	No contracture
	Followed by 1 μg. acetylcholine	32
	1 μg. acetylcholine	26
	1 ,, ,, ...	24
B.	After sarin	
	0·1 μg. acetylcholine	24
	0·1 ,, ,, ...	25
	2 μg. of decamethonium for 1·5 min.	No contracture
	Followed by 0·1 μg. acetylcholine	36
	0·1 μg. acetylcholine	20

being affected (Table II). Addition of acetylcholine to the sarin-treated muscle in the presence of previously added decamethonium showed further potentiation over and above that produced by sarin. After washing, the response elicited by acetylcholine, in the continued presence of 10^{-7} M sarin but in the absence of decamethonium, was less than the initial response after sarin.

Effect of Sarin on the Potentiation Produced by Acetylcholine Toward Choline.—The responses of the muscle to choline were observed before and after incubation of the muscle with sarin 10^{-5} M. The response was increased after incubation with sarin, but returned to the control value during the next two additions of choline to the muscle despite the continued presence of the anticholinesterase. When choline was added in the presence of a subthreshold concentration of acetylcholine, the response was comparable to that obtained immediately after incubation with sarin (Table III).

Effect of a Previous Response to Acetylcholine on the Response to Decamethonium or Choline in the Absence of and After Incubation with Anticholinesterase

In the experiments of Cohen and Posthumus (1955) the frog rectus muscle was made to contract to acetylcholine, decamethonium, and choline in that order, using frog Ringer solution to wash the tissue between contractions. After incubation with dyflos or sarin, the same procedure was repeated. By this method they observed a 1.5 to 2-fold enhancement of response towards decamethonium and a 4-fold enhancement towards choline after incubation with the anticholinesterases mentioned.

These results have been confirmed in the present experiments. However, additional experiments were performed in which contractures were also obtained by decamethonium and choline before measuring those elicited by acetylcholine. The results of representative experiments are shown in Tables IV and V. Expt. No. 1 in each table shows that the control responses obtained to decamethonium and to choline are potentiated after a contracture elicited with acetylcholine in the absence of dyflos. This potentiation was temporary, since further responses, obtained by either decamethonium or choline not preceded

TABLE III

EFFECT OF SARIN ON THE RESPONSE PRODUCED BY THE ADDITION OF CHOLINE TO ISOLATED FROG RECTUS ABDOMINIS MUSCLE. EFFECT OF A SUBTHRESHOLD CONCENTRATION OF ACETYLCHOLINE

In B the muscle was incubated with 10^{-5}M-sarin for 30 min. followed by measurement of responses in 10^{-7}M-sarin.

Dose (μg./ml.)	Contracture (mm./min.)
A. Before sarin	
1,000 μg. choline	30·0
1,000 ,, ,, ...	29·5
B. After sarin	
1,000 μg. choline	34·5
1,000 ,, ,, ...	32·5
1,000 ,, ,, ...	29·5
0·005 μg. acetylcholine for 1·5 min.	No contracture
Followed by 1,000 μg. choline	34·0
0·005 μg. acetylcholine for 1·5 min.	No contracture
Followed by 800 μg. choline ..	29·5

TABLE IV

EFFECT OF ACETYLCHOLINE ON THE CONTRACTURE PRODUCED BY DECAMETHONIUM IN THE ABSENCE OF AND AFTER INCUBATION WITH DYFLOS

In B the muscle was incubated in 10^{-6}M dyflos for one hour followed by measurement of responses in 10^{-6}M dyflos.

Expt. No.	Dose (μg./ml.)	Contracture (mm./min.)
1	Not treated with dyflos	
	21 μg. decamethonium ..	24
	21 ,, ,, ..	24
	1·25 μg. acetylcholine ..	44
	21 μg. decamethonium ..	36
	1·25 μg. acetylcholine ..	50
	10·5 μg. decamethonium ..	25
	21 ,, ,, ..	33·5
	21 ,, ,, ..	26
2	A. Before dyflos	
	21 μg. decamethonium ..	19
	21 ,, ,, ..	17
	B. After dyflos	
	21 μg. decamethonium ..	19
	21 ,, ,, ..	18
	0·15 μg. acetylcholine ..	37
	21 μg. decamethonium ..	24
	0·15 μg. acetylcholine ..	34
	14 μg. decamethonium ..	16
	21 ,, ,, ..	20·5
	21 ,, ,, ..	19

TABLE V

EFFECT OF A PREVIOUS RESPONSE TO ACETYLCHOLINE ON THE CONTRACTURE PRODUCED BY CHOLINE IN THE ABSENCE OF AND AFTER INCUBATION WITH DYFLOS

In 2B the muscle was incubated with 10^{-5}M dyflos for one hour followed by measurement of responses in 10^{-6}M dyflos.

Expt. No.	Dose (μg./ml.)	Contracture (mm./min.)
1	Not treated with dyflos	
	800 μg. choline	23
	1,000 ,, ,, ...	29
	2 μg. acetylcholine	21·5
	1,000 μg. choline	36·5
	2 μg. acetylcholine	22·5
	800 μg. choline	30
	1,000 ,, ,, ...	31·5
	1,000 ,, ,, ...	29
2	A. Before dyflos	
	1,000 μg. choline	22
	1,000 ,, ,, ...	22·5
	B. After dyflos	
	1,000 μg. choline	22·5
	0·4 μg. acetylcholine	33·5
	1,000 μg. choline	27·5
	0·4 μg. acetylcholine	32
	800 μg. choline	23
	1,000 ,, ,, ...	22

TABLE VI

EFFECT OF TUBOCURARINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS MUSCLE TO DECAMETHONIUM

In the experiments with sarin the rectus muscle was incubated for 30 min. with 10^{-5}M sarin followed by measurement of responses in 5×10^{-7}M sarin.

Expt. No.	Contracture Produced by 25 μg./ml. of Decamethonium			
	Before Sarin		With Sarin	
	Without Tubocurarine	With 2×10^{-7}M Tubocurarine	Without Tubocurarine	With 2×10^{-7}M Tubocurarine
	(mm./min.)	(mm./min.)	(mm./min.)	(mm./min).
1	18	11	18	11
2	23	14	21	13
3	19	11	20	11

TABLE VII

EFFECT OF TUBOCURARINE UPON THE RESPONSE OF THE ISOLATED FROG RECTUS MUSCLE TO ACETYLCHOLINE

In the experiments with sarin the rectus muscle was incubated for 30 min. with 10^{-5}M-sarin followed by measurement of responses in 10^{-6}M-sarin.

Expt. No.	Before Sarin			With Sarin		
	Acetylcholine Concentration (μg./ml.)		Desensitization Ratio	Acetylcholine Concentration (μg./ml.)		Desensitization Ratio
	No Tubocurarine	With 5×10^{-7}M Tubocurarine		No Tubocurarine	With 5×10^{-7}M Tubocurarine	
1	1·5	3·5	2·3	0·08	0·2	2·5
2	1·5	5·0	3·3	0·075	0·25	3·3
3	1·0	3·0	3·0	0·07	0·20	2·9
4	2·0	5·5	2·8	0·20	0·60	3·0

by a response to acetylcholine, decreased to the control value.

Expt. No. 2 in Tables IV and V shows that no potentiation over the control responses upon the addition of decamethonium and choline respectively after incubation with dyflos occurred. Subsequent measurement of a response to decamethonium or choline following a contracture elicited by acetylcholine yielded a degree of potentiation no greater than that obtained in the absence of anticholinesterase. This potentiation, like that obtained following an acetylcholine response in the absence of dyflos, faded rapidly if alternate stimulation with acetylcholine is not maintained. Similar results were obtained with other rectus muscles undergoing incubation with sarin instead of dyflos.

Effect of Sarin upon the Response to Decamethonium and Acetylcholine of the Rectus Muscle Treated with Tubocurarine, Decamethonium and Acetylcholine

A reproducible rate of contracture in response to the addition of 25 μg./ml. of decamethonium was first obtained. Then the response of the rectus muscle to the same concentration of decamethonium in the presence of 2×10^{-7} M tubocurarine was measured, followed by washing with frog Ringer solution until the initial response was again obtained. The muscle was then

incubated with 10^{-5} M sarin for 30 min. and the response to 25 μg./ml. of decamethonium again elicited in the presence of 5×10^{-7} M sarin. As in earlier experiments, the rectus muscle displayed no enhanced response after incubation with sarin. The rate of contracture to 25 μg./ml. of decamethonium was then measured in the presence of 5×10^{-7} M sarin and 2×10^{-7} M tubocurarine. If the anticholinesterase had a direct potentiating action upon receptor sites in the muscle some antagonism of the previous depression of the decamethonium response by tubocurarine, in the presence of an excess of cholinesterase inhibitor, could be expected. Table VI shows that no such antagonism was demonstrable for the rectus muscles of three frogs treated in the manner described.

In a similar experiment, the effect of sarin upon the response of curarized rectus muscle to acetylcholine was determined. The concentration of acetylcholine needed to yield a constant response was determined for each of the conditions noted in Table VII. For example, in Expt. No. 1 the presence of 5×10^{-7} M tubocurarine necessitated 2.3 times the initial concentration of 1.5 μg./ml. of acetylcholine to maintain the constant response (36 ± 1 mm./min.) obtained before treatment with sarin. The ratio of the doses required to give equal responses before and after tubocurarine is called the "desensitization ratio." After incubating with sarin, only 1/19 of the original concentration of acetylcholine (0.08 μg./ml.) was required to give the initial contracture. But after the addition of tubocurarine (5×10^{-7} M) to the medium containing the sarin, the concentration of acetylcholine required to maintain the initial contracture increased 2.5-fold over that needed in sarin alone. The other experiments shown in Table VII, carried out in the same way, agreed with Expt. No. 1—that is, they show no direct antagonism of sarin to tubocurarine over and above that due to its property of inactivating cholinesterase.

Discussion

The frog rectus abdominis muscle is believed to contain slow muscle fibres which respond to acetylcholine (and other depolarizing substances) with slow contractile changes (Brecht and Epple, 1952; Kuffler and Vaughan Williams, 1953). Such slow muscle fibre systems in frog ilio-fibularis muscle show true mechanical facilitation—that is, during a series of stimuli successive nerve volleys add increasing amounts of tension (Kuffler and Vaughan Williams, 1953).

The application of an appropriate concentration of acetylcholine (or other suitable depolarizing agent) mimics the effect of stimulating the nerve supply of such muscles (Burgen and MacIntosh, 1955). Consequently it may be postulated that the potentiation between subthreshold concentrations of one depolarizing agent and an effective concentration of another develops by a facilitatory mechanism comparable to that demonstrated for the frog ilio-fibularis muscles. This explanation would include the potentiation of the muscle response to acetylcholine by decamethonium before treatment with dyflos. It serves also to explain the other examples of potentiation developed in the presence of subthreshold concentrations of the depolarizing agents studied. In addition, the presence of anticholinesterases was found to be unnecessary for the potentiation produced by the presence of subthreshold concentrations of acetylcholine and decamethonium, as well as butyryl and succinyl choline (Fleisher, Corrigan and Howard—unpublished observations), on the response elicited by addition of effective doses of the same drugs and of choline to frog rectus muscle.

Cohen and Posthumus (1955) observed no further sensitization by decamethonium toward acetylcholine after treatment with anticholinesterases. We found a significant potentiation in the response of sarin-treated muscle to acetylcholine in the presence of a subthreshold concentration of decamethonium (Table II) over and above the increased sensitization to acetylcholine induced indirectly by inactivation of cholinesterase (Miquel, 1946). Table II also shows that the relative increase in response to acetylcholine developed in the presence of identical subthreshold concentrations of decamethonium is the same before and after sarin. If the anticholinesterase possessed the property of sensitizing the receptor sites so as to yield an enhanced response to decamethonium, then a significantly greater effect of subthreshold doses of decamethonium could have been expected after incubation with sarin. The small and temporary potentiation obtained to choline following sarin incubation (Table III) may be attributed to accumulation of endogenous acetylcholine in the presence of an anticholinesterase (Fatt and Katz, 1952).

In addition sarin showed no antagonism to tubocurarine over and above the anticurare activity derived from its anticholinesterase action. That is, the inhibitory action of tubocurarine on receptor sites as measured by its depression of the response of the rectus muscle to decamethonium

or acetylcholine was not antagonized by the cholinesterase inhibitor (Tables VI and VII). It would be expected that if sarin acted on the rectus muscle exclusively as an anticholinesterase, and tubocurarine specifically reduced the depolarizing action of applied acetylcholine (del Castillo and Katz, 1956), then the degree of desensitization to acetylcholine produced in the rectus muscle by identical partially curarizing concentrations of tubocurarine would be approximately the same with and without sarin. The results in Table VII strongly support this conclusion for the rectus muscle under the conditions tested. The direct depressant action of some anticholinesterases on receptor sites observed by other investigators has usually been found with concentrations considerably greater than those required for complete inhibition of cholinesterase activity (Eccles and MacFarlane, 1949; Kirschner and Stone, 1951; and Axelsson, Gjone, and Naess, 1957).

The consistent potentiation of the contracture of the muscle elicited by decamethonium and choline following a previous response to acetylcholine and washing, before incubation with an anticholinesterase, may be explained on the basis of a residual sensitization of receptors, or subthreshold depolarization following the response to stimulation with acetylcholine. This hypersensitivity was transient, and usually disappeared after 20 to 30 min. soaking in frog Ringer. This explanation is supported by the observation of a chemical component in slow muscle-fibre activity which can restore tension or shortening after temporary collapse (Kuffler and Vaughan Williams, 1953).

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ASSAY OF LYSERGIC ACID DIETHYLAMIDE AND ITS PASSAGE FROM BLOOD INTO THE PERFUSED CEREBRAL VENTRICLES

BY

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On the isolated rat uterus, lysergic acid diethylamide had an oxytocic action in a concentration of 2×10^{-8}; in smaller concentrations (10^{-9} to 10^{-10}), which had no stimulating effect of their own, it potentiated acetylcholine-induced contractions. This potentiating effect was made the basis for assaying minute amounts of lysergic acid diethylamide. The method was used to assay this substance in plasma of cats during its intravenous infusion at a rate of 10 μg./min./kg. During these infusions 0.4 to 2 ng./min. of lysergic acid diethylamide passed into the cerebral ventricles perfused with a salt solution of a composition resembling that of cerebrospinal fluid.

The present experiments show that minute doses of lysergic acid diethylamide augment acetylcholine-induced contractions of the isolated rat uterus, and this effect can provide the basis for a simple, sensitive method of assay of lysergic acid diethylamide. This method was used in experiments on cats during which lysergic acid diethylamide was infused intravenously, to measure the plasma concentration of the drug and to detect its appearance in the fluid with which the cerebral ventricles and the subarachnoidal space of the brain were perfused.

Apart from determinations of labelled lysergic acid diethylamide, only two methods have been used previously for the assay of this drug. Lanz, Cerletti and Rothlin (1955) used the inhibition which lysergic acid diethylamide exerts on the contractions of the rat uterus induced by 5-hydroxytryptamine, and they were able to assay amounts of 1 to 2 ng./ml. The disadvantage of this method derives from the persistence of the effect of the lysergic acid diethylamide so that 1.5 hr. have to elapse between two tests. Axelrod, Brady, Witkop and Evarts (1957) isolated the lysergic acid diethylamide from sodium chloride-saturated biological material by extraction into heptane. The lysergic acid diethylamide in the heptane extract was then returned to diluted HCl and estimated spectrofluorometrically. In this way, as little as 1 ng./ml. could be detected.

There are two reports on the plasma concentration of lysergic acid diethylamide, and one on its passage into the cerebrospinal fluid after single intravenous injections of this substance. Lanz *et al.* (1955) determined the plasma concentration in mice after injection of 35 mg./kg. Axelrod *et al.* (1957) injected smaller amounts in cats and monkeys. After the injection the concentration in the blood fell quickly, but, even 90 min. after an intravenous injection of 1 mg./kg. into a cat, the plasma concentration was 1.75 μg./g. At this time the cerebrospinal fluid contained 0.36 μg./g. which was only a little lower than the concentration in the brain, namely 0.52 μg./g. In a monkey a maximal concentration of nearly 50 ng./ml. was reached in the lumbar cerebrospinal fluid 10 min. after an intravenous injection of 0.2 mg./kg. lysergic acid diethylamide. The concentration in the cerebrospinal fluid then decreased slowly but remained about the same as that found for the unbound form of this substance in plasma. Axelrod *et al.* (1957) concluded that there was little hindrance to the passage of lysergic acid diethylamide across the blood brain barrier.

METHODS

Isolated Rat Uterus.—Albino rats weighing about 200 g. were injected intramuscularly with 1 mg./kg. stilboestrol 24 hr. before they were killed, and one uterine horn was suspended in a bath containing 5 ml. of the solution which was used by Gaddum, Peart and Vogt (1949) for the assay of adrenaline on the rat uterus. This solution contained NaCl 9, KCl 0.42, $CaCl_2$ 0.06, $NaHCO_3$ 0.50 and dextrose 0.5 g./l. of distilled water. The bath fluid was bubbled with oxygen and was maintained at 30°. The quantities of acetylcholine refer to the chloride.

Cat Experiments.—These experiments were carried out on cats which weighed 2.3 to 2.5 kg. and which were anaesthetized by the intraperitoneal injection of 35 mg./kg. of pentobarbitone sodium. The trachea was cannulated and artificial ventilation was usually administered during the experiment. The arterial blood pressure was recorded from the right femoral artery. The cerebral ventricles were perfused from a cannula implanted into the left lateral ventricle to either the cannulated cisterna or the cannulated aqueduct as described by Bhattacharya and Feldberg (1958). The solution used for perfusion was that introduced by Merlis (1940); it resembles normal cerebrospinal fluid and contains NaCl 8.1, KCl 0.25, $CaCl_2$ 0.14, $MgCl_2$ 0.11, $NaHCO_3$ 1.76, Na_2HPO_4 0.07, urea 0.13 and glucose 0.61 g./l. The rate of infusion was 0.1 ml./min. and the effluent was collected for 30 min. or 1 hr. These samples were kept at 4° until tested on the rat uterus, usually on the same day. Lysergic acid diethylamide was infused at a rate of 0.2 ml./min. into the left femoral vein using a continuous slow injector (C. F. Palmer, Ltd.). For each experiment a fresh solution of lysergic acid diethylamide in saline was prepared containing in 0.2 ml. 10 μg./kg. To estimate the lysergic acid diethylamide content of blood, 2 ml. samples of arterial blood were removed from the left femoral artery with a siliconed glass syringe, brought into a siliconed test-tube containing a small amount of heparin, and centrifuged. The plasma was removed and kept in siliconed glass vessels until diluted and tested on the rat uterus.

Results

Lysergic Acid Diethylamide on Rat Uterus

Two effects of lysergic acid diethylamide were obtained on the rat uterus. The drug sensitized the preparation to the action of acetylcholine and, in larger doses, it caused rhythmic contractions. The sensitizing effect on the acetylcholine contractions was usually obtained with 0.5 ng. to 1 ng./ml. but sometimes with as little as 0.1 ng./ml. In these experiments, acetylcholine was given every 2 min. and kept in the bath for 30 sec. The doses used varied between 0.03 and 0.05 μg./ml. and gave threshold responses. Two procedures were used for adding lysergic acid diethylamide. In the first, the same amount of lysergic acid diethylamide was given twice after washing out

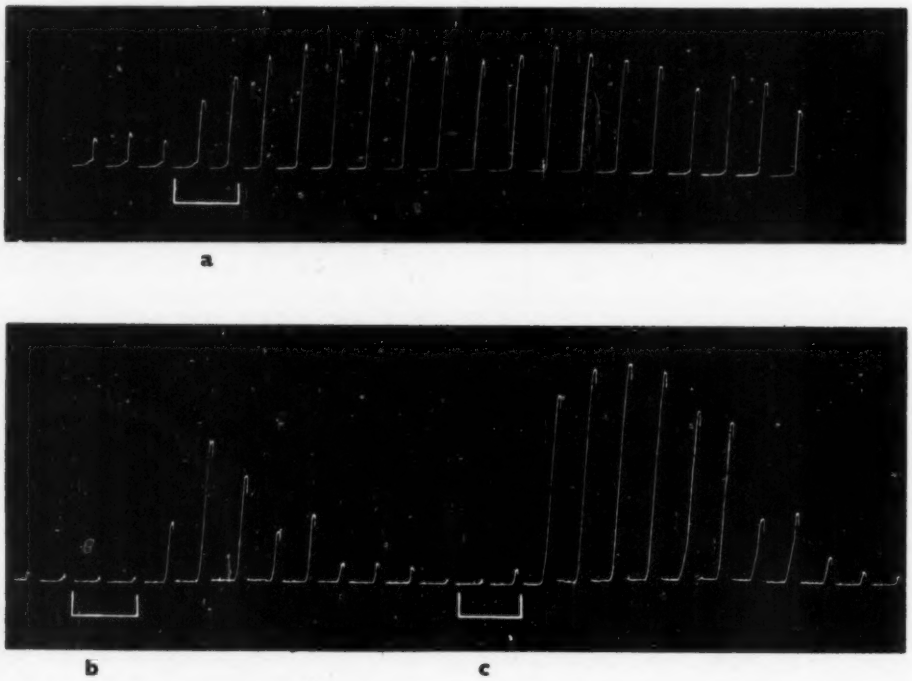

FIG. 1.—Records of contractions of the rat uterus suspended in 5 ml. bath in response to acetylcholine. Upper tracing : the effect of 0.4 ng./ml. of lysergic acid diethylamide (at **a**) on contractions produced by 0.05 μg./ml. of acetylcholine. Lower tracing: a record made with another uterus showing effect of 0.5 ng./ml. (at **b**) and of 1 ng./ml. (at **c**) of lysergic acid diethylamide on contractions produced by 0.036 μg./ml. of acetylcholine. For details about timing, see text.

two successive doses of acetylcholine, so that the time of contact of the lysergic acid diethylamide with the preparation was about 4 min. In the second, lysergic acid diethylamide was kept in the bath for 5 min. during which time no acetylcholine was given. Then after 5 min. acetylcholine was added to the bath without washing. Both drugs were washed out 30 sec. later, and the testing for acetylcholine was continued.

Fig. 1 (upper tracing) shows the long-lasting sensitizing effect of 0.4 ng./ml. of lysergic acid diethylamide (at **a**) on small contractions produced by 0.05 μg./ml. of acetylcholine using the first procedure. The lower record was obtained from another preparation and illustrates the sensitizing effect of 0.5 and 1 ng./ml. of lysergic acid diethylamide (at **b** and **c** respectively) on threshold contractions produced by 0.036 μg./ml. of acetylcholine. The effect of 1 ng./ml. was more pronounced and longer lasting than that of 0.5 ng./ml. of lysergic acid diethylamide.

The second procedure was used in the experiment shown in Fig. 2. The sensitization of the response to 0.04 μg./ml. of acetylcholine increased with increasing doses of lysergic acid diethylamide ; at **a**, 1, and at **b**, 2 ng./ml. respectively were added to the bath for 5.5 min. When the dose of lysergic acid diethylamide was increased to 20 ng./ml. (at **c**), there was not only a further augmentation of the acetylcholine responses but also rhythmic contractions appeared. This latter response is shown in the lower record. The rhythmic contractions started in the 5-min. period at **c** during which 20 ng./ml. of lysergic acid diethylamide was kept in the bath and increased during the subsequent 15 min., then gradually decreased but were still present at the end of the record about 1 hr. after washing out the lysergic acid diethyl-

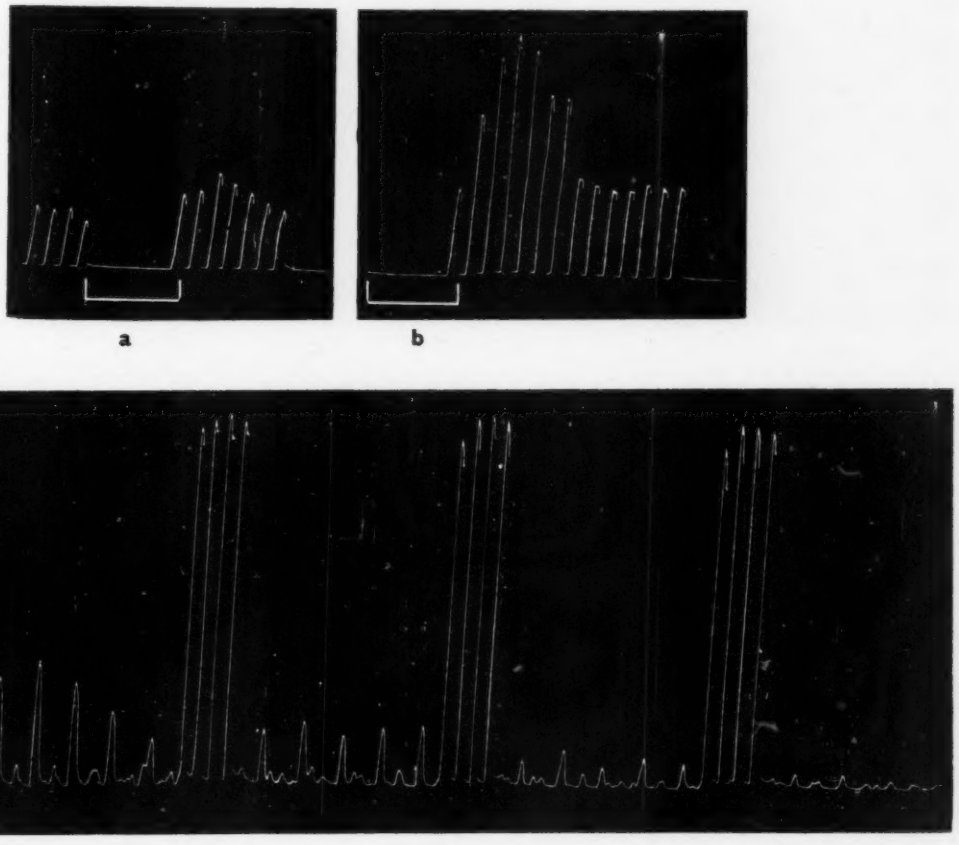

FIG. 2.—Records of the contractions of rat uterus suspended in 5 ml. bath in response to 0.04 μg./ml. of acetylcholine. At **a**, 1 ng./ml. ; at **b**, 2 ng./ml. ; and at **c**, 20 ng./ml. of lysergic acid diethylamide added to the bath for 5.5 min. In the lower tracing, the testing with acetylcholine was discontinued after each group of four acetylcholine contractions for periods of about 15 min. during which the rhythmic activity is seen.

amide. The augmentation of the responses to 0.04 μg./ml. of acetylcholine by this concentration of lysergic acid diethylamide is shown by four groups of four acetylcholine contractions. Between each group the testing of acetylcholine was discontinued for about 15 min. in order to show the rhythmic contractions. It will be seen that the augmentation of the acetylcholine responses persisted for at least 1 hr.

The potentiating effect of minute doses of lysergic acid diethylamide on the acetylcholine responses can be used as a method for the assay of lysergic acid diethylamide. Two saline solutions, which contained 260 and 606 ng./ml. lysergic acid diethylamide, when assayed without knowing their lysergic acid diethylamide contents gave estimated values of 230 and 670 ng./ml. respectively.

Since plasma influenced the acetylcholine responses of the rat uterus this fact had to be taken into account when assaying lysergic acid diethylamide in plasma. When cat plasma, which had been collected in siliconed glass tubes containing a small amount of heparin, was added in volumes of 0.1 ml. to the 5 ml. bath, the acetylcholine responses increased. Sometimes the augmentation was slight, sometimes it was pronounced, but it always differed from the effect of lysergic acid diethylamide in that the augmentation persisted only for as long as the plasma remained in the bath. When the preparation had been repeatedly treated with small doses of lysergic acid diethylamide, plasma sometimes produced its effect in volumes smaller than 0.1 ml. In one experiment, a strong effect was obtained with as little as 0.005 ml. (Fig. 3 at **b**). This effect of

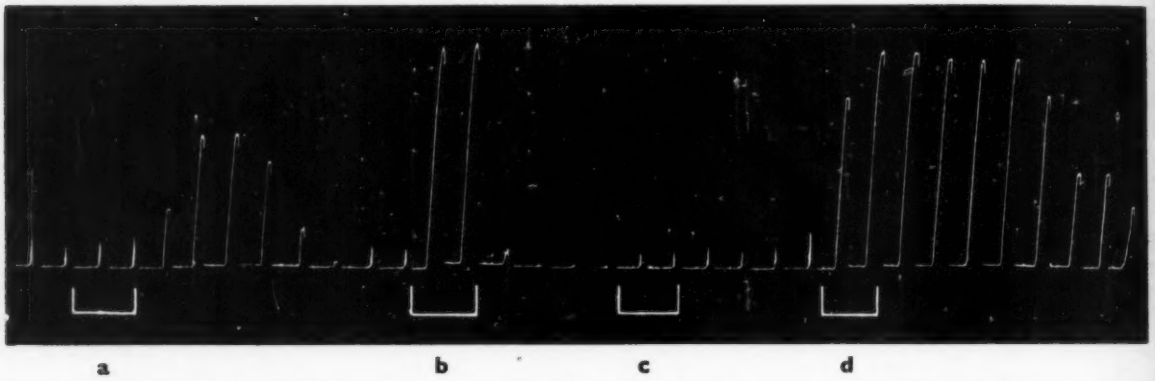

FIG. 3.—Record of the contractions of rat uterus suspended in 5 ml. bath in response to 0.036 μg./ml. of acetylcholine. At **a**, the effect of 0.5 ng./ml. of lysergic acid diethylamide. At **b**, 0.005 ml. of normal cat plasma was added to the 5 ml. bath. At **c** and **d**, 0.00125 ml. and 0.0025 ml. respectively of plasma obtained after an intravenous infusion into the cat of 10 μg./min./kg. of lysergic acid diethylamide. For details of timing, see text.

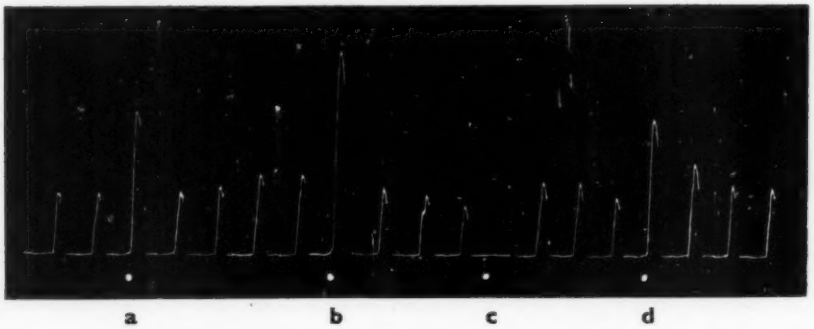

FIG. 4.—Records of the contractions of rat uterus suspended in 5 ml. bath in response to 0.025 μg./ml. of acetylcholine. At the white dots 0.1 ng./ml. (at **a** and **d**) and 0.15 ng./ml. (at **b**) of 5-hydroxytryptamine were added to the bath 30 sec. before the acetylcholine. At the white dot at **c**, 0.2 ng./ml. of 5-hydroxytryptamine given alone. For details of timing, see text.

plasma never interfered with the assay of plasma samples obtained after intravenous infusion of lysergic acid diethylamide even if the amounts of plasma that had to be added to the bath were sufficiently large to produce the short lasting augmentation. In the assay shown in Fig. 3 the volumes of plasma required were, however, so small that they did not sensitize the preparation. The effect of 0.00125 ml. of this plasma was less and that of 0.0025 ml. was more than that of 2.5 ng. of lysergic acid diethylamide which was tested before and after (not shown in Fig. 3) the plasma samples. Thus 1 ml. of this plasma contained more than 1 and less than 2 ng. of lysergic acid diethylamide.

5-Hydroxytryptamine caused augmentation of the response of the rat uterus to acetylcholine in doses too small to contract the uterus, namely 0.1 to 0.2 ng./ml. As shown in Fig. 4, the augmentation, like that produced by plasma occurred only whilst the 5-hydroxytryptamine was present in the bath. The synergism between 5-hydroxytryptamine and acetylcholine on the rat uterus has recently been observed by Barlow and Khan (1959). The augmentation produced by plasma cannot be due to 5-hydroxytryptamine since the effect of 5-hydroxytryptamine was abolished by lysergic acid diethylamide whereas that of plasma was often more pronounced after treatment of the uterus with small doses of lysergic acid diethylamide.

Intravenous Infusion of Lysergic Acid Diethylamide into Cats

An intravenous infusion of 10 μg./min./kg. of lysergic acid diethylamide into an anaesthetized and artificially-ventilated cat caused, within 10 to 20 min., a gradually increasing dilatation of the pupils; within 1 to 2 hr. the dilatation became maximal. In one cat in which the nictitating membranes were fully relaxed before the lysergic acid diethylamide infusion, they were gradually retracted during the first 10 min. of the infusion. The infusion of lysergic acid diethylamide caused a gradually-developing fall in arterial blood pressure of 20 to 60 mm. during 1 hr. and some slowing of the heart rate. These are well known effects of lysergic acid diethylamide observed on intravenous injection of single doses (Rothlin, 1957).

Plasma Concentration of Lysergic Acid Diethylamide.—In five cats the plasma of samples of blood removed after 1 or 2 hr. intravenous infusion of 10 μg./min./kg. of lysergic acid diethylamide contained between 1 and 2 μg./ml.

TABLE I

CONCENTRATION OF LYSERGIC ACID DIETHYLAMIDE IN PLASMA AND ITS OUTPUT FROM THE PERFUSED CEREBRAL VENTRICLES DURING ITS INTRAVENOUS INFUSION INTO A CAT

The bold numerals refer to the output into aqueductal, the other numerals to the output into cisternal effluent. Lysergic acid diethylamide was infused intravenously at 10 μg./min./kg.

Expt. No.	Plasma Concentration (μg./ml.) After				Output (ng./min.) During			
	1st hr.	2nd hr.	3rd hr.	4th hr.	1st hr.	2nd hr.	3rd hr.	4th hr.
1	2	2			0·6	1·2		
2	1·5	1·3			>0·9	1·5		
3	1·5	1·8			**>0·5**	**0·4**		
4	1·5	2			**1·9**	**1·4**		
5	1				**0·2**	**0·4**		
6	—	4·5	5	5	0·5	0·9	**0·8**	**<1·0**

of lysergic acid diethylamide. In one cat in which blood samples were removed 2, 3 and 4 hr. after the infusion of lysergic acid diethylamide, the plasma concentration varied between 4.5 and 5 μg./ml. These results are shown in Table I.

Appearance of Lysergic Acid Diethylamide in Cisternal and Aqueductal Effluent.—During the intravenous infusion of 10 μg./min./kg. of lysergic acid diethylamide, the substance appeared in the fluid with which the cerebral ventricles and the subarachnoidal space of the brain were perfused. There was no obvious difference in the output of lysergic acid diethylamide whether perfusion was from the lateral ventricle to the cisterna or from the lateral ventricle to the aqueduct. The output varied from 0.4 to 1.9 ng./min. This is shown in Table I. In Expt. 6 in Table I, perfusion was for the first 2 hr. from the lateral ventricle to the cisterna. The lysergic acid diethylamide output was 0.5 ng./min. during the first and 0.9 ng./min. during the second hour. The subarachnoidal space was then excluded from the perfusion by collecting the outflow from the cannulated aqueduct. During the first hour the output of lysergic acid diethylamide in the aqueductal effluent fell slightly to 0.8 ng./min. but rose in the second hour to over 1 ng./min.

These results suggest that all or nearly all the lysergic acid diethylamide in the cisternal effluent had entered the perfusion fluid on its passage through the lateral and third ventricles. It is

doubtful whether there is also a direct passage of lysergic acid diethylamide from the blood into the subarachnoidal space.

Discussion

An oxytocic effect of lysergic acid diethylamide has so far apparently been observed only on the rabbit uterus, on which the effect is slightly weaker than that of ergometrine (Rothlin and Cerletti, 1956; Rothlin, 1957). The present experiments show that lysergic acid diethylamide, in a concentration of 2×10^{-8}, had an oxytocic action also on the isolated rat uterus. In even smaller concentrations (10^{-9} to 10^{-10}), in which it had no direct stimulating effect, it potentiated the contractions produced by acetylcholine. Although lysergic acid diethylamide inhibits pseudocholinesterase (Thompson, Tickner, and Webster, 1955), it is unlikely that the potentiation can be explained by this property since the rat pseudocholinesterase is relatively insensitive to lysergic acid diethylamide and the pseudocholinesterase plays little or no part in the destruction of acetylcholine by living tissue (Shelley, 1955; Admiraal, Myers, and Houten, 1955).

The potentiating effect on the acetylcholine contractions provides a simple and sensitive method for the assay of minute amounts of lysergic acid diethylamide. Using this method of assay it could be established that lysergic acid diethylamide can pass from the blood into the fluid perfusing the cerebral ventricles during an intravenous infusion of this substance.

In previous experiments (Draškoci, Feldberg, and Haranath, 1959) the passage of adrenaline into the perfused cerebral ventricles has been examined under similar experimental conditions but with the intravenous infusion of larger amounts of adrenaline (40 μg./min./kg.). As far as it is possible to compare the results obtained with these two substances infused in different concentrations, there seems to be no great quantitative difference in the passage from the blood into the perfused cerebral ventricles. Adrenaline passed also into the perfused subarachnoidal space, and in fact in greater amounts than into the perfused cerebral ventricles. This was particularly evident from the experiments in which various parts of the subarachnoidal space were perfused without the cerebral ventricles. For lysergic acid diethylamide, no definite evidence of its direct passage from the blood into the subarachnoidal space was found, but no experiments were carried out with parts of the subarachnoidal space separately perfused during its intravenous infusion. It is therefore not possible to say whether the lysergic acid diethylamide, which Axelrod *et al.* (1957) detected in the lumbar cerebrospinal fluid of monkeys after an intravenous injection of this substance, had entered these spaces directly from the blood or by way of the cerebral ventricles.

I am grateful to Sir Charles Harington for granting me the opportunity of working in the National Institute for Medical Research, and to Dr. W. Feldberg for his advice and encouragement. The lysergic acid diethylamide used in these experiments was a gift from Messrs. Sandoz.

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Brit. J. Pharmacol. (1960), **15**, 35.

THE EFFECT OF IPRONIAZID AND IMIPRAMINE ON THE BLOOD PLATELET 5-HYDROXYTRYPTAMINE LEVEL IN MAN

BY

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Observations are reported on the blood platelet 5-hydroxytryptamine content of six patients receiving imipramine, *N*-(γ-dimethylaminopropyl)-iminodibenzyl hydrochloride. The response was a fall to a level of one-sixth of the original in three weeks, with little change thereafter. This is in sharp contrast to the action of iproniazid which caused a rise of some 200% in the blood platelet 5-hydroxytryptamine level over the same period. Imipramine in a concentration of 1 mg./ml. had no inhibitory action on 5-hydroxytryptophan decarboxylase; 8·0 μg./ml. of imipramine suppressed two-thirds of the *in vitro* uptake of 5-hydroxytryptamine (2·5 μg./ml.) by normal human platelets.

The recently developed pharmacological approach to the treatment of disordered mental states, particularly schizophrenia, with the so-called tranquillizing or ataractic drugs has undoubtedly achieved results (Brill and Patton, 1957), although the individual response is unpredictable and the mode of action is still not clear. Nevertheless, the degree of success has been sufficient to cause the adoption of a similar approach to the treatment of the other major functional psychosis, the manic-depressive syndrome. Of the two aspects of this illness, the depressive is the more frequent in the ratio of 10 to 1 and therefore calls for more urgent attack.

Iproniazid, developed for the treatment of tuberculosis, was among the first drugs to achieve improvement of mood in psychotic depression; it has been known for some time as a powerful inhibitor of amine oxidase (Zeller, Barsky, Fouts, Kircheimer and van Orden, 1952). Imipramine, *N*-(γ-dimethylaminopropyl)-iminodibenzyl hydrochloride (G.22355, Tofranil, Geigy), has more recently been claimed to be of value in the treatment of typical endogenous depressions (Kuhn, 1957, 1958; Kielholz and Battegay, 1958).

The knowledge that iproniazid was an inhibitor of amine oxidase suggested that it might cause an increase in 5-hydroxytryptamine *in vivo*, and when trials of this drug were instituted it was decided to follow the blood platelet 5-hydroxytryptamine levels. Pletscher and Bernstein (1958) have since shown that the expected increase does in fact occur. Imipramine was similarly studied to determine whether it also caused a rise of blood platelet 5-hydroxytryptamine.

METHODS

Blood was withdrawn from an arm vein into a siliconed 10 ml. syringe through a No. 20G needle. The needle was removed and 9 ml. of the blood transferred to a siliconed centrifuge tube containing 1.0 ml. of a 1.0% solution of disodium diaminoethanetetraacetate and 0.7% NaCl and mixed by slow inversion. After removal of blood for a platelet count, the tube was transferred to a chilled 15 ml. centrifuge bucket. This was prepared by placing the 15 ml. bucket without its rubber cushion inside a 50 ml. bucket containing its rubber cushion, filling the intermediate space with water, and storing the combination in a deep freeze cabinet (−15°) until required; the rubber cushion of the 15 ml. bucket was replaced immediately before use. The blood was centrifuged for 20 min. at 150 *g*. The platelet-rich plasma was withdrawn into a siliconed pointed centrifuge tube and centrifuged in a chilled bucket for 15 min. at 2,000 *g*. The button of platelets was freed from supernatant plasma and suspended in 7 ml. of physiological saline, care being taken to obtain a uniform suspension. A sample was taken for a platelet count from this suspension, which was then frozen to −15°.

Platelet counts were carried out by the method of Baar (1948). The recovery of platelets averaged 72%.

Estimation of 5-Hydroxytryptamine.—Estimations were carried out in duplicate on 3 ml. portions of the saline suspension by a fluorimetric method based on that of Udenfriend, Weissbach and Clark (1955), as modified by Brodie, Tomich, Kuntzman and Shore

(1957). In this method the suspension is brought to *p*H 10 with the addition of borate buffer, and shaken with *n*-butanol; heptane is then added to the butanol solution and the 5-hydroxytryptamine extracted from the solvent mixture into 0.1 N HCl. It is estimated in the Aminco-Bowman Spectrophotofluorimeter using exciting light of wavelength 295 mμ and measuring the fluorescence in the range 330 to 340 mμ.

The Locarte fluorimeter, Pattern LMF/2 (Laurence, 1957), does not incorporate monochromators, and filters had to be employed. Since it seemed likely to be difficult to obtain filters giving good transmission of the fluorescence with complete cut-off of the exciting light, use was made of the fact that the fluorescence shifts into the visible with a peak at 550 mμ if the 5-hydroxytryptamine solution is made strongly acid (Udenfriend, Bogdanski and Weissbach, 1955). The filters used have been, on the primary side, Chance OX7+2 cm. thickness of $NiSO_4$ solution (437.5 g./l.), and, on the secondary side, Chance OY3.

Other modifications have involved the retention of the buffer and solvent volumes given in the original paper of Udenfriend *et al.* (1955b) and the use of 0.5 M formate buffer *p*H 4 for the final extraction. There was a considerable reagent blank, equivalent to five to ten times the fluorescence from the original formate buffer; this appeared to be due to butanol. The procedure adopted to reduce this interference to a minimum was to measure the fluorescence of the formate buffer extracts against a reagent blank prepared by passing water through the extraction procedures. To the solutions, volume about 1.5 ml., 0.1 ml. of concentrated HCl was then added. This addition caused some reduction of the reagent blank fluorescence, which was brought back to the previous scale reading by increasing the sensitivity of the instrument. The standard and unknown 5-hydroxytryptamine fluorescences were then measured, the net values being obtained by deducting the readings obtained before adding HCl. A linear relationship between scale reading and 5-hydroxytryptamine concentration was thus obtained. Each group of estimations carried out included pairs of reagent blanks and 5-hydroxytryptamine standards. In order to reduce systematic errors, the duplicates from one platelet suspension were always estimated in separate groups.

Fluorimetric methods of estimation of 5-hydroxytryptamine have come in for criticism, particularly in relation to brain tissue. A comparison of the results obtained by the technique described above with those of other workers is therefore given in Table I.

The mean value for normal subjects obtained with the simple fluorimetric method is practically identical with the mean value obtained with the spectrophotofluorimeter. They are 25% higher than the mean value obtained by the bioassay technique; the difference, though probably significant, is not large and should not lead to grave errors in serial studies.

The Effect of Imipramine on the Estimation of 5-Hydroxytryptamine.—Another source of error may be the presence of imipramine in the blood. It was early noted that, in mixed aqueous solutions, 3 μg./ml. of imipramine could make the fluorimetric estimation of 5-hydroxytryptamine impossible, since imipramine itself fluoresced at *p*H 4 and the fluorescence disappeared on adding HCl. Herrmann and Pulver (personal communication) found that the blood imipramine concentration in animals receiving higher than therapeutic doses was not measurable by the only method available, that is, it was less than 1 μg./ml.

However, even the simplified procedure involves two steps, (1) the separation of the platelets from the blood and (2) the butanol extraction. In Table II, figures are given for the effect of varying concentrations of imipramine on the estimation of 5-hydroxytryptamine in an aqueous mixture of the two compounds subjected to butanol extraction. The

TABLE I

NORMAL BLOOD PLATELET 5-HYDROXYTRYPTAMINE LEVELS: COMPARISON OF RESULTS OF DIFFERENT WORKERS AND METHODS

Figures in brackets are standard deviations, except those marked with an asterisk, which are limits including 95% of observations.

Authors	Method	Description of Subjects	Number	Mean Platelet 5-Hydroxytryptamine of Blood (μg./ml.)
Feldstein, Hoagland, and Freeman (1959)	Spectrophotofluorimeter	Normal, male	16	0·19 (±0·08)
Hardisty and Stacey (1955)	Bioassay: rat uterus	Normal, male and female	35	0·16 (±0·06)
This paper	Filter fluorimeter	Normal, male	20	0·20 (±0·05)
Weiner and Udenfriend (1957)	Spectrophotofluorimeter	General hospital patients, representative selection, sex unspecified	94	0·23 (0·05–0·49)*
This paper	Filter fluorimeter	Mental hospital patients, male	86	0·24 (0·07–0·50)*

TABLE II

THE EFFECT OF IMIPRAMINE ON THE ESTIMATION OF 5-HYDROXYTRYPTAMINE EXTRACTED FROM AQUEOUS MIXTURES

Aqueous 5-hydroxytryptamine solutions (3·0 ml. samples) were extracted by standard procedure. Percentage recovery was based on parallel extracts of solutions in the absence of imipramine. Figures are means of four estimates, and refer to free base.

5-Hydroxytryptamine Concentration (μg./ml.)	Imipramine Concentration (μg./ml.)	5-Hydroxytryptamine as Percentage of that Extracted from a Solution Containing no Imipramine
0·144	7·5	101
0·144	3·8	100
0·144	1·9	95
0·036	30·0	17
0·036	7·5	85
0·036	1·9	89

higher of the two concentrations of 5-hydroxytryptamine employed is 75% of the normal mean level for platelet suspensions, the lower about 20%. Somewhat surprisingly, since imipramine is a base, the procedure appears to remove it, at any rate up to a concentration eight times the suggested upper limit in blood.

If the imipramine in the blood were equally distributed between the various components, the separation of the platelets, first from the erythrocytes and subsequently from the plasma, would reduce the concentration of imipramine in the saline suspension to one-four-hundredth of that in the original blood. However, since platelets are known to absorb amines, it did not appear wise to make this assumption. Therefore, in experiments on the effect of imipramine on the uptake of 5-hydroxytryptamine by normal platelets, a control was added to give a figure for the effect of imipramine on the estimation of endogenous 5-hydroxytryptamine from platelets. The results are summarized in Table III; they show that imipramine did not interfere with the estimation of 5-hydroxytryptamine in platelets.

Uptake of Exogenous 5-Hydroxytryptamine by Platelets.—The experimental technique was based on that of Born, Ingram, and Stacey (1958). To reduce natural variation, blood for this series of experiments was drawn only from four normal volunteers and no individual's platelets were subjected to the same set of experimental conditions twice. A volume of 22 ml. of blood was taken as previously described and mixed with 2 ml. of anticoagulant; of the platelet-rich plasma, isolated in the standard manner, 1.5 ml. was added to each of four siliconed pointed centrifuge tubes containing 0.5 ml. saline, and either imipramine,

TABLE III

THE EFFECT OF IMIPRAMINE ON THE ESTIMATION OF 5-HYDROXYTRYPTAMINE IN PLATELETS

Three parts of platelet-rich plasma were mixed with one part of saline suspension or drug in saline suspension; mixtures were incubated for 60 min. at 37°. Estimations were in duplicate on each mixture. Imipramine concentrations are as free base; 5-hydroxytryptamine percentages are means.

Imipramine Concentration (μg./ml.)	Number of Experiments	5-Hydroxytryptamine in Presence of Imipramine (Expressed as % 5-Hydroxytryptamine Estimated in Absence of Imipramine)
0·44	3	93
1·77	6	115
7·10	2	105

5-hydroxytryptamine or 5-hydroxytryptamine+imipramine. The final concentrations of imipramine used were 0.5, 2.0, and 8.0 μg./ml.; the 5-hydroxytryptamine concentration was 2.5 μg./ml. except in one set of experiments, where it was 10.0 μg./ml. The final dilutions of the compounds were in saline. After mixing carefully but thoroughly a sample was withdrawn for a platelet count. The tubes were loosely covered and placed vertically in a thermostat at 37° for 60 min., being shaken at sixty double oscillations (6 cm. amplitude) per min. They were then centrifuged under the standard conditions. Since 5-hydroxytryptamine was present in two of the tubes the methods described by Born *et al.* (1958) for the removal of as much plasma as possible were used; the platelets were then resuspended in 5 ml. of saline of which 2 ml. portions were taken for duplicate estimations of 5-hydroxytryptamine. A sample for a platelet count was taken immediately after resuspension.

Effect of Imipramine on 5-Hydroxytryptophan Decarboxylase. — The manometric technique of Davison and Sandler (1958) was employed.

Liver Function Tests.—Determinations of serum bilirubin, thymol turbidity, and alkaline phosphatase activity were carried out by standard methods (King and Wootton, 1956).

RESULTS

Except where otherwise stated, this study refers to male patients who were receiving either imipramine or iproniazid for therapeutic purposes.

Effect of Iproniazid on Platelet 5-Hydroxytryptamine.—Four patients received this drug in tablet form; the dose was raised over the course of 4 days to 225 mg./day. Two patients were taken off the drug after 11 and 13 days; the other two continued to receive it for 33 and 38 days.

The response of the blood platelet 5-hydroxytryptamine level to the drug, calculated as the percentage change from the mean for the pre-treatment values, is given in Fig. 1. Where drug treatment continued for an adequate length of time increases of 200% and upwards were observed. On stopping the drug a return to normal levels occurred.

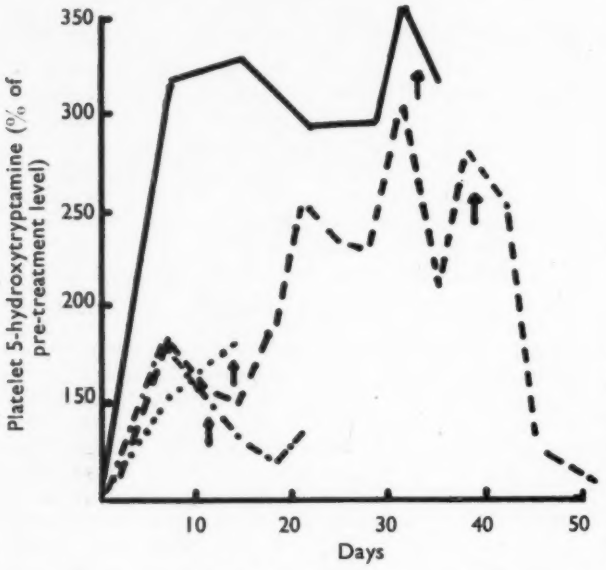

FIG. 1.—The effect of iproniazid on platelet 5-hydroxytryptamine in man. Each line represents one patient. Arrows indicate termination of treatment.

Effect of Imipramine on Platelet 5-Hydroxytryptamine.—Six patients received a standard course of imipramine hydrochloride, commencing with the parenteral therapy recommended for severe cases of depression but passing over to oral therapy at the maximum level of 300 mg./day by the 9th day. The detailed dosage schedule is given in Table IV. Subsequently the dose was adjusted to meet the patients' requirements and ranged from 150 to 300 mg./day.

There was no great variation in response between individuals when this was calculated in terms of the percentage change from the pre-treatment level. The mean values have therefore been given (Fig. 2). Imipramine caused a fall in platelet 5-hydroxytryptamine to 17% of the pre-treatment level after 3 weeks. There was no further significant change up to 6 weeks. A sharp rise in one patient to 50% of the pre-treatment level after 6 weeks was subsequently found to correlate with the patient's refusal to take the drug over a period of 10 days.

TABLE IV

DAILY DOSAGE SCHEDULE FOR IMIPRAMINE

Figures refer to mg. of imipramine hydrochloride. The total daily doses were distributed between three administrations.

Day	Injection mg.	Oral mg.	Total mg.
1	75	0	75
2	100	0	100
3	125	0	125
4	100	50	150
5	75	100	175
6	50	150	200
7	25	200	225
8	0	250	250
9	0	300	300

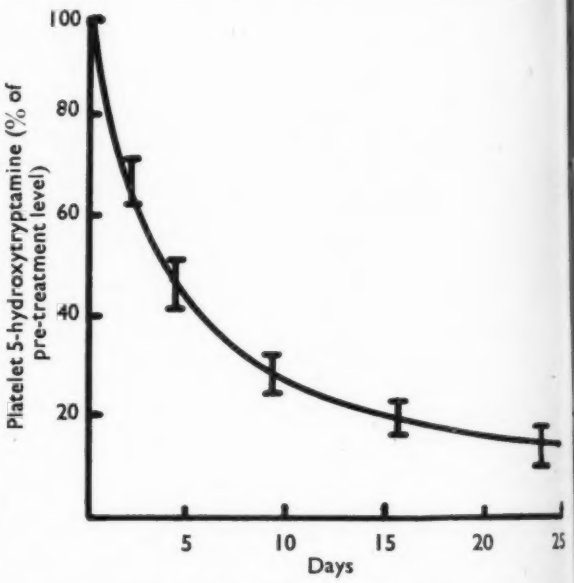

FIG. 2.—The effect of imipramine on platelet 5-hydroxytryptamine in man. Points represent mean percentage of pre-treatment level for six patients, with standard deviations of means.

Observations on the Mode of Action of Imipramine.—Investigation of the action of imipramine on 5-hydroxytryptophan decarboxylase gave negative results: there was no measurable inhibition of enzyme activity at an imipramine concentration of 3×10^{-3} M (1 mg./ml.).

In one patient, the level of excreted 5-hydroxyindoleacetic acid was investigated during a course of therapy. There was no fall in 5-hydroxyindoleacetic acid excretion over a

period of 3 weeks. It has recently been pointed out, however (Milne, 1959), that a fall in the level of excretion is not conclusive proof of decreased synthesis but may also be due to reduced urinary clearance.

In preliminary observations, comparison of the uptake of 5-hydroxytryptamine by normal platelets and by those from patients who had been on continuous imipramine therapy gave equivocal results. It was therefore decided to study the effect of imipramine on normal platelets *in vitro* in the first instance, in order to permit adequate controls with known concentrations of the compounds to be made.

The results of this series of experiments are given in Table V. The uptake of 5-hydroxytryptamine from a diluted plasma containing 2.5 μg./ml. averaged about 300% of the endogenous platelet level. An imipramine concentration of 8.0 μg./ml. reduced the uptake to one-third, and even 0.5 μg./ml. appeared to cause some inhibition. A four-fold increase in 5-hydroxytryptamine concentration markedly reduced the extent of the inhibition, though it raised the uninhibited uptake of 5-hydroxytryptamine by only 40%.

Liver Function Tests on Patients Receiving Iproniazid and Imipramine.—A frequent check is kept on the possibility of liver damage in patients receiving iproniazid (Pare and Sandler, 1959). Liver function tests were done not on the six male patients receiving imipramine but on nine female patients receiving much the same dosage. The results are summarized in Table VI. The percentage of results of these tests above the recognized normal upper limits (Varley, 1954) averaged 22% for iproniazid and 4% for

TABLE V

THE UPTAKE OF 5-HYDROXYTRYPTAMINE BY HUMAN PLATELETS IN THE PRESENCE OF IMIPRAMINE

Platelet-rich plasma (1·5 ml.) was added to 5-hydroxytryptamine and imipramine in saline (0·5 ml.) and incubated for 60 min. at 37° with shaking (60 × 6 cm. double oscillations per min.). Initial 5-hydroxytryptamine contents are the means of values for saline controls and imipramine controls, as in Table III. Drug concentrations are as free base.

5-Hydroxytryptamine Concentration (μg./ml.)	Imipramine Concentration (μg./ml.)	5-Hydroxytryptamine Content (ng./10^8 Platelets)			Percentage Inhibition of Uptake Due to Imipramine
		Initial	After Incubation without Imipramine	After Incubation with Imipramine	
1·1	7·1	82	307	169	61
		40	160	76	70
		90	314	172	63
					Mean 65
1.1	1·77	79	283	168	56
		74	266	177	46
		123	291	200	54
					Mean 52
1·1	0·44	62	229	222	4
		48	361	173	60
		72	271	224	24
					Mean 29
4·3	1·77	56	305	273	13
		69	385	308	24
		115	399	428	−11
					Mean 9

TABLE VI

RESULTS OF LIVER FUNCTION TESTS ON PATIENTS RECEIVING IPRONIAZID AND IMIPRAMINE

Test	Percentage of Tests Giving Figures above Recognized Upper Limit of Normal for Bed Patients		Percentage of Tests Giving Figures Higher than: Bilirubin, 0·5; Thymol Turbidity, 2·0; Alkaline Phosphatase, 8·0	
	Iproniazid	Imipramine	Iproniazid	Imipramine
Bilirubin	17	0	35	15
Thymol turbidity	17	0	92	0
Alkaline phosphatase	33	12	54	19
All tests	22	4	60	11

imipramine. These limits refer to bed patients; the upper limits for normal individuals (and possibly for ambulant mental patients) might be somewhat lower. Varley (1954) gives an upper limit of 2.0 units for the thymol turbidity test in normal subjects; King and Wootton (1956) state that the great majority of normal bilirubin values are below 0.5 units. The percentage above these arbitrarily fixed and more stringent upper limits was 60% for iproniazid and 11% for imipramine.

DISCUSSION

The findings on iproniazid confirm those of Pletscher and Bernstein (1958). The observed fall in platelet 5-hydroxytryptamine in patients receiving imipramine is of interest. Aspects of the pharmacology of this compound have been studied (Bradley and Key, 1959; Mörsdorf and Bode, 1959; Domenjoz and Theobald, 1959), but the findings do not appear to relate directly to the present observation. Himwich (1959) quotes a personal communication by Costa that imipramine increases brain 5-hydroxytryptamine in the rabbit.

The results of the experiments *in vitro* suggest a possible mode of action of imipramine *in vivo*; they do not provide independent confirmation of the validity of the fluorimetric technique nor, assuming this to be valid, do they necessarily account for the quantitative aspects of the findings *in vivo*. An attempt to confirm the present findings using a bioassay technique is obviously desirable, but cannot be undertaken in this laboratory. The exact concentrations of neither imipramine nor 5-hydroxytryptamine in human plasma are known; a value of less than 1.0 μg./ml. has been quoted for imipramine (Herrmann and Pulver, personal communication) and figures of 0.002 and of less than 0.0001 μg./ml. have been given for 5-hydroxytryptamine (Humphrey and Jaques, 1954; Armin and Grant, 1957). The concentrations chosen for the experiments *in vitro* were considerably higher than the concentrations occurring *in vivo*. It appears to be the ratio of the concentrations which is important; however, no extrapolation can be attempted with so many factors unknown. Using lower concentrations of both compounds *in vitro* is not necessarily going to provide the answer, since *in vitro* uptake appears to stop within 75 to 90 min. (Born *et al.*, 1958), whereas *in vivo* the changes would hardly be significant in the first 24 to 48 hr.

Our results suggest that imipramine resembles reserpine in inhibiting the uptake of 5-hydroxytryptamine by platelets (Brodie *et al.*, 1957), but beneath this similarity there lie a number of differences in detail. Reserpine in therapeutic dosage reduces the platelet 5-hydroxytryptamine level to zero in 24 hr., whereas imipramine requires the same number of days to achieve its maximum effect; an equilibrium state appears then to be attained, rather than a complete inhibition. The experiments *in vitro* suggest that, unlike reserpine (Shore, Carlsson and Brodie, 1957), imipramine does not set free the endogenous 5-hydroxytryptamine. With imipramine, depletion may involve competition rather than destruction.

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OXIDATION OF 5-HYDROXYTRYPTAMINE AND RELATED COMPOUNDS BY *MYTILUS* GILL PLATES

BY

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Ho nogenates of gill plates of *Mytilus edulis* L. used oxygen when 5-hydroxytryptamine was added. The oxidation of 5-hydroxytryptamine was not due to the presence of an amine oxidase, but to that of an enzyme that catalysed the oxidation of other 5-hydroxyindoles (5-hydroxytryptophan, bufotenine). The oxidation was cyanide-sensitive, but was not inhibited by iproniazid. In the reaction a yellowish-brown substance was formed. The occurrence of an amine oxidase in the anterior retractor muscle of the byssus and in the digestive gland was confirmed.

A number of observations suggest that in molluscs 5-hydroxytryptamine acts as a hormone or mediator; these have been reviewed by Welsh (1955). In *Mytilus edulis*, the anterior retractor muscle of the byssus is inhibited by 5-hydroxytryptamine in very low concentrations (Twarog, 1954). Aiello (1957) reported that 5-hydroxytryptamine had a stimulating action on the ciliary movements in the gill epithelium of *Mytilus* and that extracts of gill plates had a similar effect.

Amine oxidase, a catalyst of the biological inactivation of 5-hydroxytryptamine, occurs in many molluscs. In *Mytilus*, it was not demonstrated in earlier studies (Blaschko and Hawkins, 1952), but more recently Blaschko and Hope (1957) found amine oxidase in homogenates of both the digestive gland and the anterior retractor muscle of the byssus, but no amine oxidase activity was found in the gill plates. Since in the work on *Mytilus* gill plates 5-hydroxytryptamine had not been used as substrate, it was decided to find out if gill plate extracts oxidized 5-hydroxytryptamine.

METHOD

Fresh specimens of *Mytilus edulis* L. were usually dissected immediately after arrival at our laboratory at Oxford. When necessary animals were kept aerated in sea water at 4°. Homogenates were usually prepared without adding fluid; they were dialysed, with frequent changes, against 0.067 M-sodium phosphate buffer of *p*H 7.4 for 2 to 4 hr.; dialysing against the phosphate buffer was then continued without changes overnight.

For the manometric experiments, two different types of conical manometer vessels were used. One had a capacity of 16 to 18 ml., the other 7.5 to 8.5 ml. In the larger vessels the volume of the reaction mixture was 2.0 or 3.0 ml., in the smaller vessels it was 0.7 ml. The substrates were tipped in from the side bulb to give an initial concentration of 0.5×10^{-2} M. The gas phase was either oxygen or, in all later experiments, air. The temperature of the manometer bath was 25°.

5-Hydroxytryptamine creatinine sulphate, 5-hydroxy-DL-tryptophan and iproniazid were obtained from Roche Products. We are grateful to Dr. R. B. Barlow and Dr. I. Khan for the sample of bufotenine hydrochloride.

RESULTS

When a dialysed homogenate of *Mytilus* gill plates was incubated with 0.5×10^{-2} M-5-hydroxytryptamine, a slow but steady uptake of oxygen occurred. No similar uptake of oxygen was found when the homogenate was incubated with tryptamine. In the presence of 5-hydroxytryptamine, a yellowish-brown colour soon developed and increased in intensity as the reaction proceeded.

To establish whether the oxidation of 5-hydroxytryptamine was due to the presence of an amine oxidase in the gill plate homogenates, a second experiment was carried out in which the homogenate was incubated with 5-hydroxytryptamine in the presence of either 10^{-3} M-iproniazid or 10^{-2} M-potassium cyanide, and also without any other substance. To ensure that the iproniazid was effective in inhibiting any amine oxidase present, all flasks were pre-incubated at 25° for 30 min. before tipping. In this experiment, the flask containing iproniazid showed the same

oxygen uptake, and the same development of brown colour, as that to which no inhibitor had been added. However, both the oxygen consumption and the development of colour were entirely suppressed in the presence of cyanide.

That the oxygen consumption in the presence of 5-hydroxytryptamine was due to an enzyme was supported by the observation that no uptake of oxygen occurred when 5-hydroxytryptamine was added to a gill plate homogenate after this had been heated in a boiling water bath for 30 min.

The observations reported made it unlikely that the catalyst involved in the oxidation of 5-hydroxytryptamine was amine oxidase. First, there was an absence of oxidation of tryptamine, usually a good substrate for the study of amine oxidase, and second, there was the lack of inhibition by iproniazid and also the inhibition by cyanide.

In another experiment, both phenethylamine and tyramine were incubated with the gill plate homogenate. Neither amine was oxidized. With tyramine, observations were continued for 180 min. to establish whether there was a phenolase present which required a prolonged incubation of a monophenolic amine; no oxygen uptake occurred within the period of observation.

The findings suggested that the oxidation of 5-hydroxytryptamine by the gill plate homogenates depended upon the presence of the phenolic hydroxyl group in position 5 on the indole nucleus. An experiment was therefore carried out in which both 5-hydroxytryptamine and 5-hydroxytryptophan were incubated. Oxygen uptake occurred not only in the flask incubated with 5-hydroxytryptamine, but also in the flask which contained 5-hydroxytryptophan; the rate of oxidation with the latter was a little slower than with 5-hydroxytryptamine. As with 5-hydroxytryptamine, a brown colour developed when 5-hydroxytryptophan was oxidized, but the rate of development of colour was also a little slower than with 5-hydroxytryptamine.

That the enzymic reaction was, in fact, characteristic of 5-hydroxyindoles was supported by the observation that bufotenine was also oxidized. In Fig. 1 an experiment is shown in which the rates of oxidation with 5-hydroxytryptamine were compared with 5-hydroxytryptophan and with bufotenine. Development of colour was seen with each of these substances.

Although there had been no oxygen uptake with tyramine, dopamine (3,4-dihydroxyphenethylamine) was oxidized; its rate of oxidation in two experiments was slightly less than that of 5-hydroxytryptamine. The oxidation of dopamine was also accompanied by a development of colour, but the greyish-black colour was in marked contrast with the yellowish-brown one formed when the 5-hydroxyindoles were oxidized.

In the earlier experiments, the gas phase during incubation was 100% oxygen, but it was found that there was no significant difference in the rate of the enzymic reaction when the manometers contained air. For this reason, in all later experiments, the gas phase was air. The independence of the rate of the enzymic reaction from the partial pressure of oxygen is also in contrast to what is known of the properties of amine oxidase: the latter is less active in air than in 100% oxygen (Kohn, 1937).

On high-speed centrifugation the activity was found to be retained in the supernatant fluid. In a preliminary experiment, a homogenate was centrifuged at 18,000 *g*; the enzymic activity of the supernatant fluid did not significantly differ from that of the original homogenate.

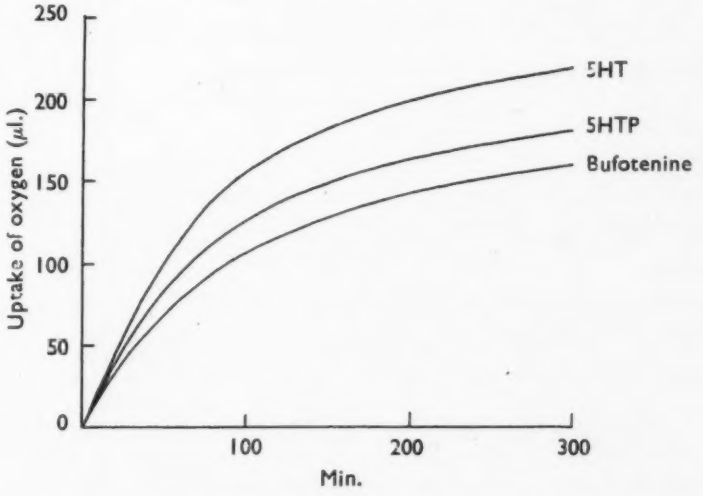


FIG. 1.—Oxidation of 5-hydroxytryptamine, 5-hydroxytryptophan and bufotenine by a preparation from gill plates of *Mytilus edulis* at 25° in air. Each flask contained 1.8 ml. of a resuspended sediment from a 25–55% ammonium sulphate precipitation. Substrate concentrations: 0.5×10^{-3} M. Abscissa: time in min. Ordinate: µl. oxygen used.

A further purification of the oxidase responsible for the enzymic reaction was carried out on a larger batch of homogenate as follows:

Ten dozen specimens of *Mytilus* were dissected to give 129 g. of gill plates. This material was stored at −12° overnight. After homogenizing, it was dialysed for 2 hr. against distilled water and for a further 2 hr. against the phosphate buffer. The homogenate was then centrifuged for 1 hr. at 40,000 r.p.m. in the angle head No. 40 of the Spinco ultracentrifuge. The supernatant fluid from this centrifugation—100 ml.—was used as starting material for two ammonium sulphate precipitations. In the first experiment, 50 ml. was used and three ammonium sulphate precipitates and a final supernatant fluid were obtained. The first sediment was the precipitate from adding ammonium sulphate to bring saturation to 25%; the second from 25 to 55%, and the third from 55 to 100%. Each of these sediments was suspended in 5 ml. of phosphate buffer and they, as well as the final supernatant fluid, were dialysed against the buffer for two hours.

No activity was present in the final supernatant fluid, but all three sediments contained enzymic activity. Most of the activity was recovered in the precipitate from the 25 to 55% saturation. The recoveries, in terms of the supernatant fluid obtained by high-speed centrifugation which had been the starting material for the ammonium sulphate precipitation, were as follows:

High-speed supernatant (starting material)	800	μl. O_2/hr.
Sediment from 0 to 25% saturation	80	„ „
„ „ 25 to 55% „	600	„ „
„ „ 55 to 100% „	437	„ „

The second batch of the same high-speed supernatant fluid was used for another ammonium sulphate precipitation at 30, 60, and 90% saturation; in this experiment the protein content of the various fractions was also determined.

The results summarized in Table I show that the greater part of the activity was recovered in the precipitate from the 30 to 60% saturation. The specific activity of this fraction was 2.09, as compared with 0.64 for the high-speed supernatant fluid, the starting material for the ammonium sulphate precipitation. In other words, the sediment from the 30 to 60% saturation contained 77% of the enzymic activity of the starting material, and at the same time a 3.3-fold purification had been achieved. The overall purification from the original homogenate must have been considerably greater, but this fraction still contained too much fibrous material to make the determination of protein useful.

The more highly purified enzyme preparations were used to find out how much oxygen was consumed in the oxidation reaction. For this experiment, 1.35 ml. of the resuspended sediment from the 30 to 60% ammonium sulphate precipitation, plus 0.45 ml. phosphate buffer, was incubated with 5.0 μmoles of 5-hydroxytryptamine (total reaction volume 2.0 ml.). The oxidation reaction, which proceeded rapidly at first but with a slowly declining rate later, was followed for 5 hr. It came to a standstill after 4 hr., when 100.5 μl. oxygen corresponding to 4.5 μmoles of oxygen had been taken up. In other words, 0.9 moles of oxygen had been consumed per mole of substrate added.

TABLE I

ENZYMIC ACTIVITY AGAINST 5-HYDROXYTRYPTAMINE, AND PROTEIN CONTENT OF THE HIGH-SPEED SUPERNATANT FLUID OBTAINED FROM HOMOGENATES OF THE GILL PLATES OF *MYTILUS EDULIS* AND OF FRACTIONS OBTAINED BY AMMONIUM SULPHATE PRECIPITATION

Protein content determined according to Layne (1957). One enzyme unit (E.U.) is defined as the amount of enzyme that causes uptake of 1 μl. oxygen in 30 min. The readings for the final supernatant were too small to allow an accurate measurement of enzymic activity and protein content.

Fraction	Ammonium Sulphate Percentage Saturation	Volume of Fraction ml.	E.U./ml.	Protein mg./ml.	Specific Activity (E.U./mg. Protein)	Total Enzyme Units
High-speed supernatant ..	—	37·5	8·9	13·8	0·64	334
Sediment I	0–30	6·7	4·4	30·0	0·15	29
„ II	30–60	7·5	34·3	16·4	2·1	257
„ III	60–90	12·7	6·4	3·4	1·9	81
Final supernatant	—	36	1·4 (?)	0·14 (?)	—	—

The most active sediment from the first ammonium sulphate precipitation, that from the 25–55% saturation, was used for a comparison of the rate of oxidation of 5-hydroxytryptamine and L-dopa (β-3,4-dihydroxyphenyl-L-alanine). With 0.63 ml. of the fraction in each flask and a total reaction volume of 0.7 ml., the rates of oxidation were:

	After 15 min.	After 30 min.
With 5×10^{-3} M-5-hydroxytryptamine	8.5 μl. O_2	19 μl. O_2
With 5×10^{-3} M-L-dopa ...	12 μl. O_2	26 μl. O_2

The experiment of Fig. 1, in which the rate of oxidation of different 5-hydroxyindoles is shown, was also carried out using the sediment from the 25–55% saturation with ammonium sulphate.

A few experiments were carried out on other *Mytilus* tissues. In one experiment with 37 specimens, the anterior retractor muscles of the byssus were dissected (weight of muscles 4.0 g.) and a dialysed homogenate in phosphate buffer was prepared (total volume of homogenate 10 ml.). Of this 1.6 ml. was used in each manometer flask. Upon incubation for 1 hr. with 10^{-2} M-tryptamine and 0.5×10^{-3} M-5-hydroxytryptamine, 12.5 μl. oxygen was consumed with tryptamine and 11.5 μl. oxygen with 5-hydroxytryptamine. The corresponding figures after 2 hr. incubation were 23.5 μl. and 21 μl. respectively. Here also a brown colour appeared, but, in contrast to the experiments with the gill plate homogenates, a strong bluish-white fluorescence was seen, particularly in the flask incubated with tryptamine. This experiment confirmed the occurrence of amine oxidase in the anterior retractor muscle, as already reported by Blaschko and Hope (1957). Both amines were also oxidized by a homogenate of the posterior retractor muscle of the byssus.

A homogenate was also prepared of the digestive glands of *Mytilus* (1 part of fresh tissue plus 2 parts of phosphate buffer); this homogenate also contained parts of the intestine and other adjoining tissues. Each manometer flask contained 1.6 ml. of the dialysed homogenate. Both tryptamine and 5-hydroxytryptamine were oxidized. In the same experiment, 10^{-3} M-iproniazid was also tested. The amounts of oxygen used in the first hour of incubation were:

With tryptamine	36 μl. oxygen
„ 5-hydroxytryptamine	19 μl. „
„ tryptamine plus iproniazid ...	1 μl. „
„ 5-hydroxytryptamine plus iproniazid	9.5 μl. „

In the experiment, the oxidation of tryptamine was more strongly inhibited by iproniazid than that of 5-hydroxytryptamine, suggesting that the oxidation of tryptamine was only due to amine oxidase but that the oxidation of 5-hydroxytryptamine was due in part to an iproniazid-resistant enzyme. Thus, it is possible that the digestive gland contains not only amine oxidase, but also the catalyst of the oxidation of 5-hydroxytryptamine found in the gill plates.

Homogenates of the gill plates, the gonads and the digestive glands of *Chlamys* (*Pecten*) *opercularis* were also prepared. Tryptamine was readily oxidized by all three homogenates, but an oxidation of 5-hydroxytryptamine occurred only with the gill plate homogenate.

Discussion

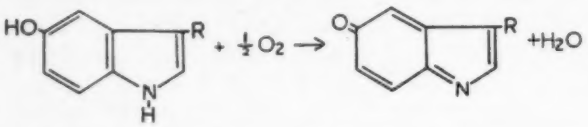
These experiments show that gill plates of *Mytilus edulis* contain an enzyme that oxidizes 5-hydroxytryptamine.

The absence of any oxygen uptake with tryptamine, phenethylamine and tyramine, the lack of inhibition by iproniazid, and the sensitivity to cyanide are all properties which show that the enzyme responsible for the oxidation is not amine oxidase. This view is supported by the observation that other 5-hydroxyindoles, 5-hydroxytryptophan and bufotenine, are similarly oxidized. All these compounds are characterized by the presence of the phenolic hydroxyl group in position 5 on the indole nucleus.

There is good reason to believe that the enzyme is of the phenolase type. First, there is the inhibition of the enzymic reaction by cyanide. Second, there is the fact that both dopamine and dopa are oxidized, the latter by a partly purified preparation of the enzyme. The lack of any uptake of oxygen with tyramine suggests that the enzyme is not a monophenoloxidase. It must be borne in mind that 5-hydroxytryptamine and other 5-hydroxyindoles are not strictly comparable to the monophenols; they are substituted derivatives of *p*-aminophenol, and should therefore be more similar to the *p*-diphenols.

It has recently been shown by Mansour (1958) that 5-hydroxytryptamine has an affinity for the phenol oxidase of the liver fluke, *Fasciola hepatica*, and that of the Harding-Passey mouse melanoma. Both enzymes are inhibited by 5-hydroxytryptamine. There was no oxidation of 5-hydroxytryptamine by the *Fasciola* enzyme, but with the melanoma Mansour's data show a very slight oxidation of 5-hydroxytryptamine. However, the chief difference between these preparations and that containing the *Mytilus* gill plate enzyme is that the latter rapidly oxidizes the 5-hydroxyindoles.

The nature of the enzymic reaction catalysed has not been studied. The most likely reaction is the formation of a quinone-imine, as already suggested by Blaschko and Philpot (1953), thus:



This reaction would require 0.5 moles of oxygen per mole of substrate. In our experiments, the total oxygen uptake was nearer one mole of oxygen per molecule of substrate; this could either be interpreted as due to a further oxidation of the product of the enzymic reaction, or to the accumulation of hydrogen peroxide, or to peroxidatic reactions in which the latter was used up.

An alternative possibility should not be forgotten: the introduction of a second phenolic hydroxyl group in the indole nucleus. There is no evidence to suggest any particular position for such a group; it may be mentioned that Carlisle (1956) has discussed the occurrence of an *ortho*-dihydroxytryptamine in the pericardial organ of Crustacea.

The relatively rapid action of a phenolase type of enzyme of molluscan origin on 5-hydroxytryptamine and related substances is of interest. It has often been discussed whether melanin-like pigments can arise from tryptophan, and it seems that the reaction here described makes it possible that pigments may arise by the oxidation of a 5-hydroxyindole. The colour of the incubation mixture was different from that produced by dopa or dopamine; it was yellowish-brown, and it may be mentioned here that we have seen a similar colour when 5-hydroxytryptamine was oxidized by silver oxide.

Whether 5-hydroxytryptamine has a regulatory function in the ciliary epithelium of the gill plates is unknown. The work of Aiello (1957), who observed an effect of 5-hydroxytryptamine on the ciliary rhythm, has already been mentioned. There is evidence of an antagonism of 5-hydroxytryptamine and acetylcholine in the molluscan heart and in the anterior retractor muscle of the byssus. It is known that in the gill plates acetylcholine has an effect on the ciliary rhythm and that the gill plates contain all the catalysts for the synthesis as well as the inactivation of acetylcholine (Bülbring, Burn and Shelley, 1953; Milton, 1959). In the light of these findings, the presence in the gill plates of a catalyst for the inactivation of 5-hydroxytryptamine is of particular interest.

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THE EFFECT OF PRECURSORS OF NORADRENALINE ON THE RESPONSE TO TYRAMINE AND SYMPATHETIC STIMULATION

BY

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Previous observations have shown that the effects of sympathetic stimulation and of tyramine were absent in the organs of animals treated with reserpine, but that they were restored by an infusion of noradrenaline. Observations are described showing that an infusion of adrenaline did not restore the pressor action of tyramine in the cat or in the rat, but that in the rat the pressor action was restored by an infusion of dopamine, or of (−)-dopa, or of *m*-tyrosine, or of phenylalanine. Observations are also described showing that the effect of postganglionic stimulation of the fibres to the nictitating membrane and to the iris was restored by an infusion of dopamine or of (−)-dopa; it was restored less well by an infusion of noradrenaline. An infusion of noradrenaline did not restore the action of tyramine on the denervated iris or on the denervated vessels of the cat's foreleg. An infusion of noradrenaline appeared to increase the effect of sympathetic stimulation of the hypogastric nerves to the uterus of the virgin cat about as much as an infusion of adrenaline. An infusion of noradrenaline restored the constrictor action of nicotine on the perfused vessels of the rabbit ear.

The pressor action of tyramine was shown by Carlsson, Rosengren, Bertler, and Nilsson (1957) to be absent in animals treated with reserpine, and we observed (Burn and Rand, 1958b) that it was restored by an intravenous infusion of noradrenaline. We also found that in normal dogs (not treated with reserpine), or in the perfused dog hindleg, the vasoconstrictor action of stimulation of the lumbar sympathetic chain was increased by an intravenous infusion of noradrenaline (Burn and Rand, 1960).

We have now carried out experiments to see if similar effects followed the infusion of adrenaline and of the precursors of noradrenaline, and have tested their effect on stimulation of sympathetic fibres to organs other than the blood vessels.

METHODS

In treating animals with reserpine a solution of reserpine 5 mg./ml. was prepared in a solution of ascorbic acid 20%. Cats received 0.6 ml. on two successive days; male rats of weight 200 g. received 0.3 ml. on two successive days. Observations were made on the blood pressure in the spinal preparation of cats and in the rats prepared as described by Muscholl and Vogt (1957), in which the brain and spinal cord of the rat are destroyed by pithing (Shipley and Tilden, 1947), and the rat receives 1 to 2 mg. atropine. In making intravenous infusions a motor-driven syringe (C. F. Palmer London, Ltd.) was used and a polythene tube was inserted into a vein other than the vein which was cannulated for injections. In the rat all infusions were made for 25 min. In the cat the duration varied from 18 to 30 min.

In experiments on the virgin cat uterus, the cat was anaesthetized with chloralose, after which the abdomen was opened and the cat was eviscerated. The ligament between the right ovary and the kidney was divided and a thread was tied to the ovary and connected to a lever writing on the drum. Sometimes stimulation was applied to both hypogastric nerves and sometimes to the right nerve only, using electrodes of a pattern already described (Burn and Rand, 1960). The nerves were dissected below the inferior mesenteric ganglion and were irrigated through the electrode-holder by a slow stream of oxygenated Krebs solution. The abdominal cavity was filled with Locke solution at 37° and maintained from a slow drip. Rabbit ears were perfused by the method described by Burn (1952) using the outflow recorder of Stephenson (1948).

RESULTS

Restoration of the Action of Tyramine.—Observations on the restoration of the pressor

TABLE I

INCREASE OF PRESSOR ACTION OF TYRAMINE IN SPINAL PREPARATIONS OF CATS TREATED WITH RESERPINE

Dose of tyramine=2 mg.

Substance Infused	Amount Infused	Pressor Response (mm. Hg)		Increase	Mean
		Initial	After Infusion		
Noradrenaline	0·1 mg.	20	48	28	19
		10	30	20	
		18	42	24	
		18	22	4	
	0·5 ,,	12	70	58	48
		10	52	42	
		8	70	62	
		20	60	40	
		2	46	44	
		18	64	46	
		19	65	46	
		22	72	50	
Adrenaline . .	0·5 ,,	38	20	18	7
		12	20	8	
		32	32	0	
		6	22	16	
		12	32	18	
		16	32	16	
Dopamine . .	5 ,,	10	34	24	13
		20	26	6	
		16	26	10	
	10 ,,	26	72	44	34
		24	48	24	

action of tyramine were made in the cat and in the rat. The results for a series of experiments in the cat are given in Table I, in which the effect of infusing noradrenaline, adrenaline, and dopamine is seen. Adrenaline had little or no effect. This is illustrated in Fig. 1 in which a series of injections of 2 mg. tyramine was given before and after the intravenous infusion of 0.5 mg. adrenaline, and of 0.5 mg. noradrenaline, repeated twice. The pressor action of tyramine was increased only after the infusion of noradrenaline, and not after the infusion of adrenaline.

The increase in the pressor action after the infusion of noradrenaline was greatest for the first injection after the infusion, and then rapidly disappeared. This is shown in Fig. 2 in which the mean results of eight experiments are shown; from these it appears that even the second injection had little more effect than the injection before the infusion.

The effect of dopamine was more lasting, as is shown in Fig. 2, although the amount of dopamine infused was 10 mg. as compared with 0.5 mg. noradrenaline, that is to say was less than an equipressor amount.

Observations in rats have been summarized in part in Table II, where the increase in the pressor action of tyramine has been expressed, not in terms of the height of the rise, but in terms of the area of the pressor response recorded on the drum. Thus the first line of Table II gives the results of three experiments in which 5 μg. noradrenaline was infused. In the first of these the area of the pressor response to the first injection of 0.5 mg. tyramine after the infusion was 9.5 times as great as it was before the infusion. Table II shows that dopamine when given in only 10 times the dose was more effective than noradrenaline. The action of dopamine and of (−)-dopa was much more lasting than that of noradrenaline, as is shown in Figs. 3 and 4.

We were not successful with tyrosine; it was dissolved with difficulty, and a trial with the N-acetyl derivative of tyrosine was also ineffective. However, an infusion of 2 mg. meta-tyrosine (having the –OH in the *meta* position in the ring) was effective in increasing the pressor action of tyramine, though rather less so than 2 mg. (−)-dopa.

TABLE II

RATIO OF PRESSOR RESPONSE TO TYRAMINE BEFORE AND AFTER INFUSION IN THE PITHED RAT TREATED WITH RESERPINE

The response was taken as the area of the rise of pressure on the kymograph.

Substance Infused	Amount Infused	Ratio, Taking Response Before Infusion=1
Noradrenaline . .	5 μg.	9·5, 8·4, 4·9
	10 ,,	12·3, 24·7, 7·3
	20 ,,	12·4, 15·9
Dopamine	50 ,,	16·4
	100 ,,	24·6
	200 ,,	37·0
(−)-Dopa	1 mg.	8·8, 6·4
	2 ,,	8·6
(±)-*m*-Tyrosine . .	2 ,,	6·4
(−)-Phenylalanine	25 ,,	9·6
(−)-Adrenaline . .	20 μg.	0·3, 3·2
(−)-N-Acetyl Tyrosine . .	10 mg.	0·25
(−)-Tyrosine . .	1·6 ,,	0·9
	3·0 ,,	0·5

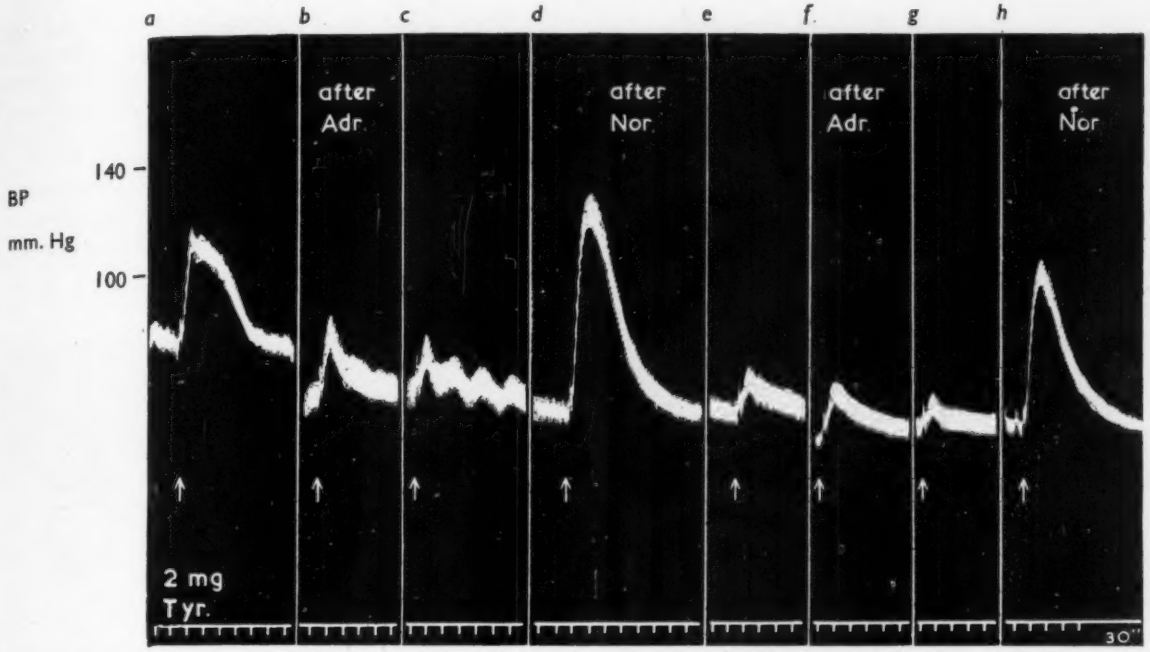

FIG. 1.—Blood pressure in spinal preparation of reserpine-treated cat. 2 mg. tyramine injected at each arrow. Between (*c*) and (*d*), and between (*g*) and (*h*), infusions of 0.5 mg. noradrenaline, after which the responses to tyramine were increased. Between (*a*) and (*b*), and between (*e*) and (*f*), infusions of 0.5 mg. adrenaline, which did not increase the responses.

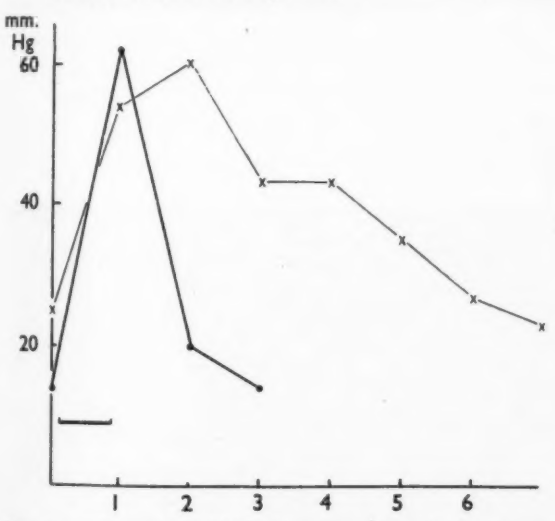

FIG. 2.—Mean rise of blood pressure in spinal preparation of reserpine-treated cat due to injection of 2 mg. tyramine. Black circles show increase after infusion (marked by horizontal line) of 0.5 mg noradrenaline in 8 cats. The increase was evident with the first injection of tyramine after the infusion, but had almost disappeared with the second injection. Crosses show increase after infusion of 10 mg. dopamine (2 cats). This increase persisted for several injections. Abscissae: successive injections of tyramine. Ordinates: rise in blood pressure (mm.Hg).

Finally we observed that an infusion of 25 mg. phenylalanine increased the pressor action of tyramine, having a prolonged effect.

Effects on Sympathetic Stimulation. — The observations on the effect of a noradrenaline infusion on sympathetic stimulation which have been made so far have been confined to vasoconstriction. We therefore turned to the iris of the cat's eye and to the nictitating membrane. An example of an experiment is given in Fig. 5. At the beginning, stimulation of the postganglionic fibres had no effect on the diameter of the pupil, and had a very small effect on the nictitating membrane. An intravenous infusion of 10 mg. dopamine was then given during a period of 15 min. About 7 min. after the end of the infusion the postganglionic fibres were stimulated again, using the same stimulus as before. The pupil dilated 5 mm. and the contraction of the nictitating membrane was 16 mm. on the drum. Stimulation at intervals during the next 40 min. continued to elicit similar large responses.

In one experiment (Fig. 6) a comparison was made between the effect of dopamine, noradrenaline, and (−)-dopa in restoring the response to sympathetic stimulation in the reserpine-treated cat. After the response of the iris had been restored by an infusion of 8 mg. dopamine,

injections of 4 mg. tyramine were given so that both the effect of sympathetic stimulation and of tyramine on the iris became very small. Noradrenaline was then infused during 20 min.; this increased the effect of tyramine greatly, but had much less effect on sympathetic stimulation. After further injections of tyramine the effect of an infusion of 30 mg. (−)-dopa was determined. It increased the effect of both tyramine injections and of sympathetic stimulation, though the effect of sympathetic stimulation was not increased as much as it had been by the infusion of dopamine.

We also observed that the effect of sympathetic stimulation on the iris was increased after giving an infusion of adrenaline.

The observation that an infusion of noradrenaline had a greater effect in increasing the response of the iris to tyramine than it had in increasing the

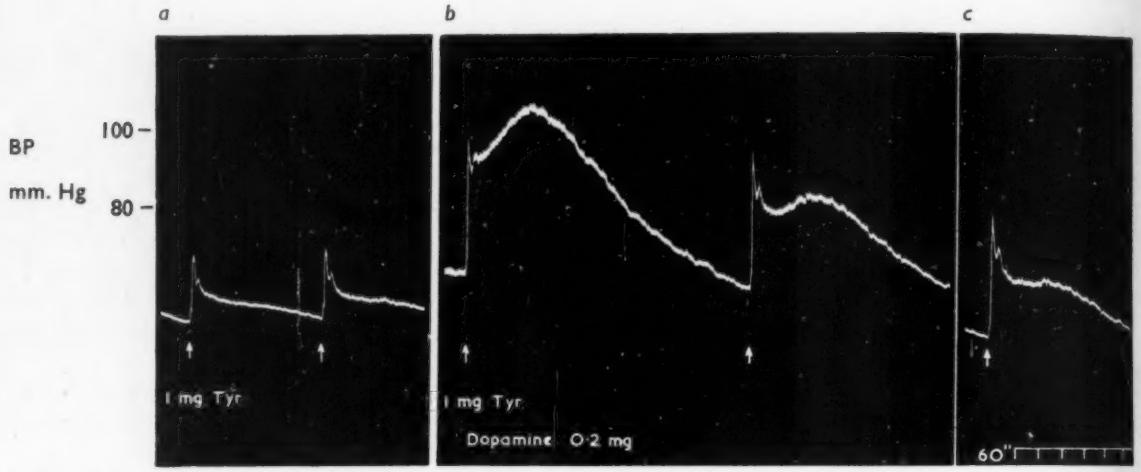

FIG. 3.—Blood pressure of pithed, reserpine-treated rat. Injections of 1 mg. tyramine into right jugular vein at each arrow. Between (*a*) and (*b*) 200 μg. dopamine was infused at 8 μg./min. into the left jugular vein. (*b*) The response to the 1st and 2nd injections of tyramine after the infusion of dopamine, and (*c*) the 3rd injection.

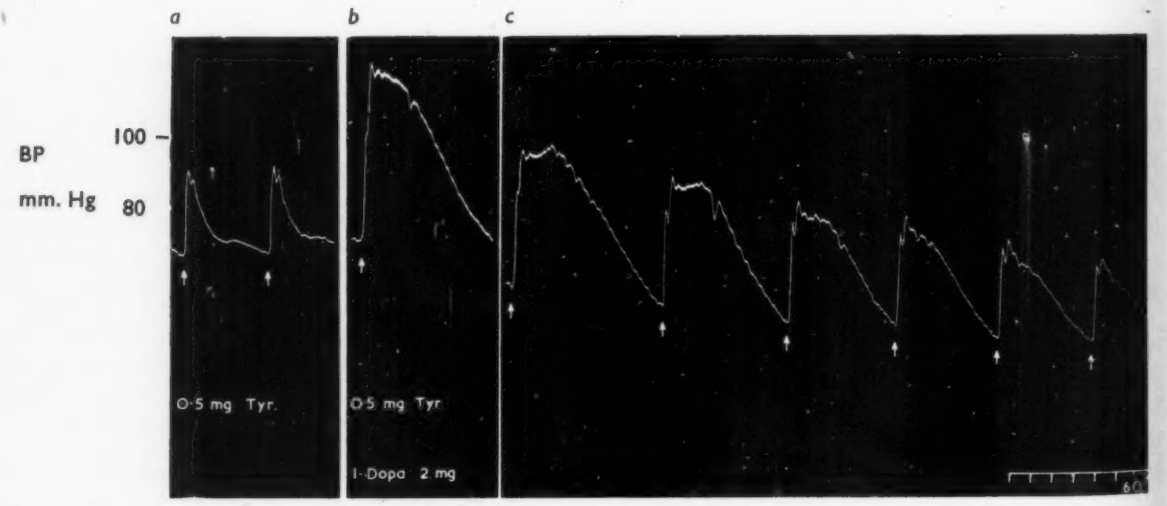

FIG. 4.—Responses to 0.5 mg. of tyramine in pithed, reserpine-treated rat (*a*). Between (*a*) and (*b*), 0.5 mg. of (−)-dopa was infused. The enhanced responses to tyramine after the infusion of (−)-dopa are shown in (*b*) and (*c*). Note that the response to the seventh injection of tyramine after the infusion of (−)-dopa was still greater than the response before the infusion.

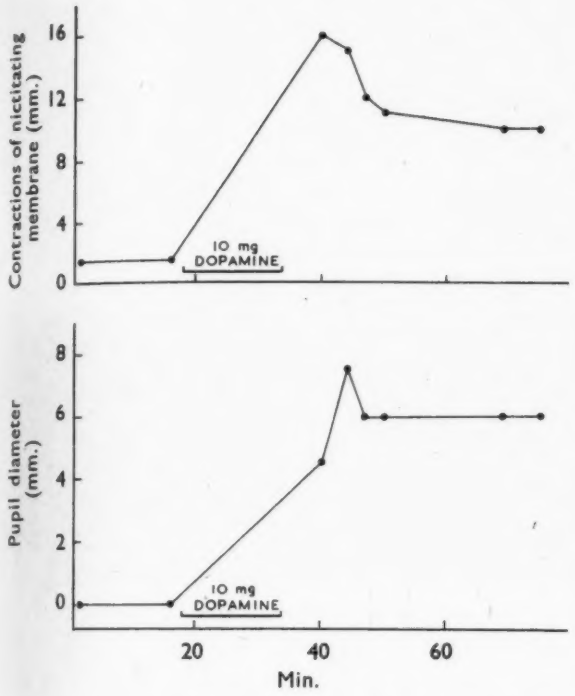

FIG. 5.—Reserpine-treated cat. Upper graph shows contractions of the nictitating membrane (mm. on kymograph record). The lower graph shows dilatation of the pupil measured directly. Stimulation of the postganglionic fibres leaving the superior cervical ganglion for 20 sec. with 5 mA. square wave pulses, 2 msec. duration, 25/sec. After the infusion of 10 mg. dopamine the responses were greater.

response to sympathetic stimulation was also made in experiments on the spleen. Before the infusion of noradrenaline was made, stimulation of the splenic nerves (in the presence of atropine) failed to cause any contraction of the spleen, and an injection of tyramine was also ineffective. After an infusion of 0.5 mg. noradrenaline, stimulation of the splenic nerves caused a very slight contraction of the spleen, while an injection of tyramine caused a large contraction.

Cat Uterus.—We made observations in which we compared the effect of an infusion of noradrenaline with that of an infusion of adrenaline on the inhibitory response to stimulation of the hypogastric nerves of the uterus of the virgin cat. An example of such a comparison is given in Fig. 7, in which the infusion of 100 μg. noradrenaline and the infusion of 100 μg. adrenaline increased the effect of sympathetic stimulation almost equally.

Effect of Infusion in Denervated Tissues.—Since noradrenaline is almost absent in denervated tissues (Euler and Purkhold, 1951; Burn and Rand, 1959), and since tyramine has no action in these tissues (Burn, 1932a), we carried out experiments to see if the infusion of noradrenaline would restore the response to tyramine. We denervated the right iris in two cats by removing the superior cervical ganglion, and after two weeks we determined the effect of an infusion of 1 mg. noradrenaline on the response of the iris to an intravenous injection of tyramine. In neither experiment did the infusion restore the response to tyramine, although the dose injected was as large as 4 mg. The pupil did not dilate at all, though the left pupil dilated maximally.

We made two experiments on the denervated vessels of the foreleg, after removal of the stellate ganglion of one side two weeks previously. The result in one of these is shown in Fig. 8, in which the changes in volume of the normally innervated foreleg, recorded by a plethysmograph, are seen at the top. The middle record is that of the denervated foreleg. The injection of 2 mg. tyramine caused constriction in the normal foreleg, and a slight passive dilatation in the denervated foreleg. These responses remained unchanged after the infusion of 1 mg. noradrenaline. The second experiment gave a similar result.

Infusion of Noradrenaline in the Rabbit Ear.—Finally experiments were made in the perfused rabbit ear in which nicotine normally has a constrictor effect. This constriction is absent if the ear is taken from a rabbit which has been treated with reserpine (Burn and Rand, 1958a), and we wished to know if the constrictor response could be restored by an infusion of noradrenaline. Fig. 9 is taken from an experiment of this kind. At the beginning the injection of 10 μg. nicotine acid tartrate into the fluid perfusing the ear caused only an increase in outflow. During the night noradrenaline was added to the fluid perfusing the ear in a concentration of 0.1 μg./ml. On the next morning a series of four injections of 10 μg. nicotine acid tartrate had a small but increasing constrictor effect as shown by the fall in outflow, and then the injection of 20 μg. caused a large constriction. Thereafter two injections to 10 μg. nicotine were each without effect. It appeared that the infusion of noradrenaline had restored some constrictor action of nicotine, but that after the larger dose of nicotine further doses were then ineffective.

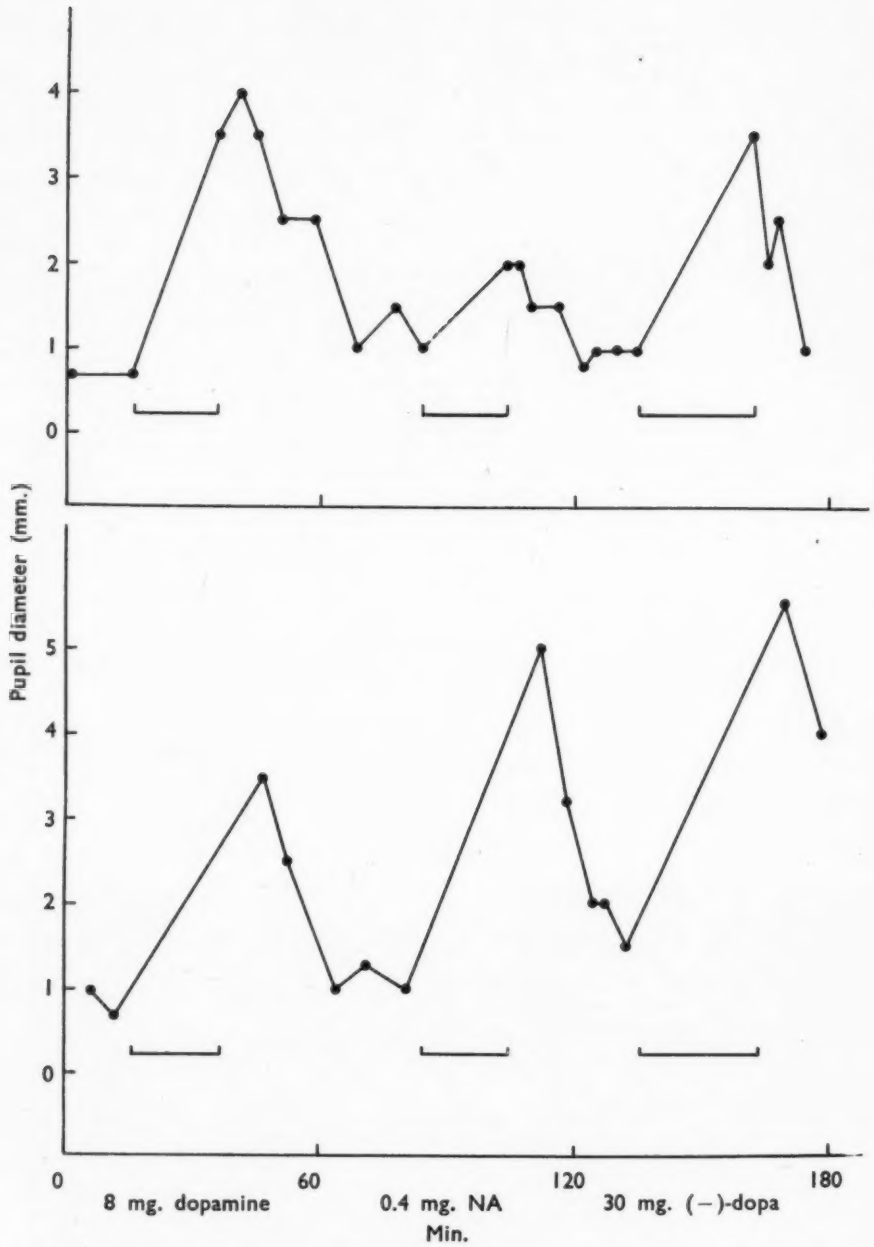

FIG. 6.—Reserpine-treated cat. Upper graph shows dilatation of the pupil in mm produced by postganglionic sympathetic stimulation. Lower graph shows dilatation produced by injection of tyramine (4 mg.). An infusion of noradrenaline (0.4 mg.) was less effective than an infusion of dopamine (8 mg.) or of (−)-dopa (30 mg.) in increasing the response to stimulation, but not less effective in restoring the response to tyramine.

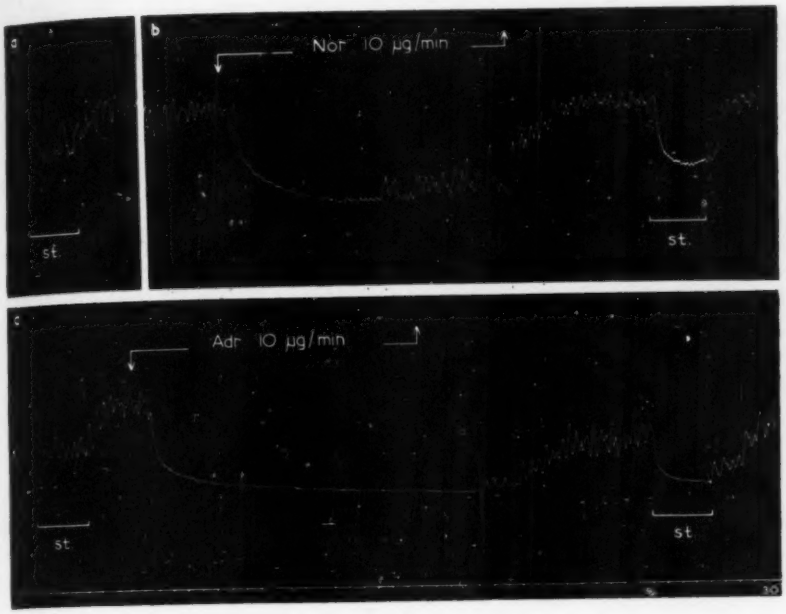

FIG. 7.—Right horn of uterus of virgin cat *in situ*. Right hypogastric nerve stimulated (St) for 2 min. with 20 mA. square wave pulses, 2 msec. duration, 25/sec. After an infusion of 100 µg. of noradrenaline, stimulation produced a greater and more complete inhibition than before. This increased response was only temporary, and about 30 min. later there was only a partial inhibition on stimulation. Then, after an infusion of 100 µg. adrenaline, stimulation was again more effective.

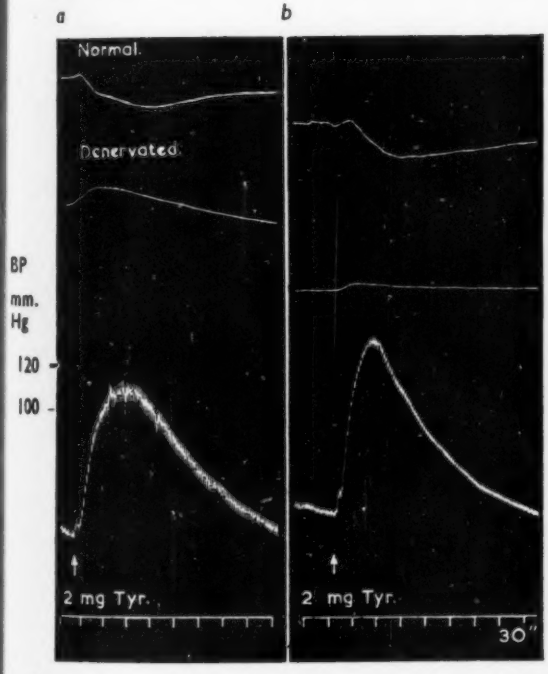

FIG. 8.—Normal (upper) and denervated (lower) foreleg plethysmograph records from cat in which right stellate ganglion had been removed 28 days previously. Spinal preparation. Tyramine (2 mg.) had no constrictor action on the vessels of the denervated foreleg (*a*) initially, or (*b*) after the infusion of 1 mg. noradrenaline.

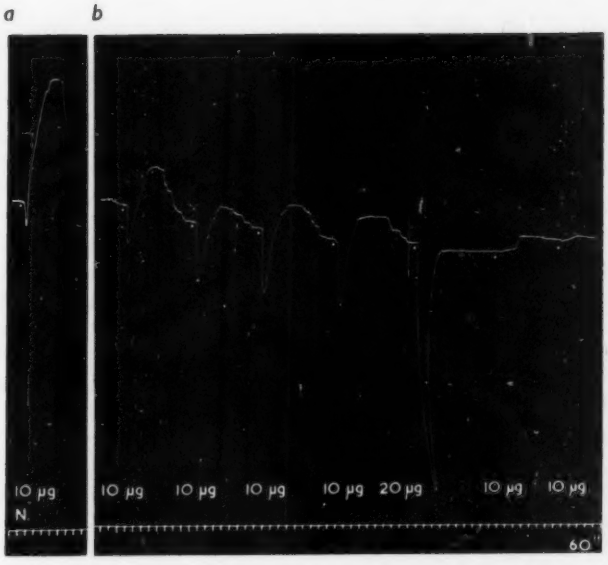

FIG. 9.—Perfused ear from reserpine-treated rabbit. In (*a*) 10 µg. nicotine acid tartrate produced dilatation. Noradrenaline (10^{-7}) was added to the perfusing solution overnight between (*a*) and (*b*). Then, during perfusion with fresh Locke solution, nicotine acid tartrate 10 µg. and 20 µg. produced constriction. After the injection of the larger dose the constrictor action of nicotine was lost.

Discussion

The observations described support our earlier suggestion (Burn and Rand, 1958b) that there is a store in the neighbourhood of sympathetic nerve endings which can take up noradrenaline from the blood stream and that the effect of sympathetic stimulation and the effect of an injection of tyramine and related amines depends on the size of the store. The earliest observations of this kind were made by Burn (1932a and b), but more recently efforts have been made to detect the uptake of catechol amines by chemical analysis of the tissues. Thus Raab and Gigee (1955) observed that the heart muscle of the dog took up noradrenaline and adrenaline after intraperitoneal injection of these substances; they recorded a 12-fold increase of noradrenaline and an even greater increase of adrenaline. However, the amounts they injected into a dog of 10 kg. were 38 mg. noradrenaline together with 75 mg. adrenaline. v. Euler (1956) infused amounts of noradrenaline between 2.5 and 7.8 μg./kg./min. into cats for over 30 min., and gave intraperitoneal injections of noradrenaline and adrenaline up to 2 mg./kg. He failed to observe any increase in the catechol amine content of the heart, the spleen, the liver, the kidney, and skeletal muscle. Recently Muscholl (private communication) has failed to observe any increase of noradrenaline in the heart muscle of a rat previously treated with reserpine after the intravenous infusion of amounts of noradrenaline of the order of 14 μg. which were sufficient to restore the pressor action of tyramine.

These failures to observe an increase in the tissues after the infusion of noradrenaline make it important to recall the precise observations which have been made. These are that organs with a sympathetic innervation contain noradrenaline which can be extracted and measured, and that this extractable noradrenaline is absent in the organs when the animal is treated with reserpine. The organs normally respond to sympathetic stimulation and to tyramine, but after treatment with reserpine they no longer do so. Their response is, however, restored by an infusion of noradrenaline, and, as we have now shown, also by an infusion of dopamine, or of (−)-dopa, or of meta-tyrosine or of phenylalanine. We have seen indications that dopamine is indeed more efficient in restoring the effect of sympathetic stimulation than is noradrenaline itself.

It is clear from studies of the restoration of the tyramine response that it is a very partial restoration. That is to say in the normal animal repeated injections of tyramine can be given for many hours without much diminution in the size of the response. In the reserpine-treated animals, after the infusion of noradrenaline, two or three injections of tyramine are sufficient to cause the restored response to disappear. The response to sympathetic stimulation appears to persist for periods of an hour or so, but we have as yet no evidence that it persists longer. Quite small amounts of noradrenaline are sufficient to restore the pressor action of tyramine; thus in one experiment the injection of 10 μg. noradrenaline into a reserpine-treated cat was enough to cause an increase, and amounts such as 0.25 mg. in a dog produced a considerable fall in the threshold for sympathetic vasoconstriction.

These observations suggest that much smaller amounts of noradrenaline can be shown to be effective by our physiological tests than can be demonstrated to have accumulated by chemical tests. It is likely that there are several stages between uptake of noradrenaline from the blood and incorporation into granules such as Blaschko and his colleagues have demonstrated in the adrenal medulla (Blaschko, 1959). Incorporation into granules of noradrenaline taken from the blood may indeed not occur at all, for what is present in the granules may be the result of synthesis only. But that the increase in the effect of tyramine or of sympathetic stimulation or of nicotine (in the rabbit ear vessels) as a result of an infusion of noradrenaline is due to retention of noradrenaline in some form and in some situation we think is most likely. In Fig. 9 an infusion of noradrenaline restored the constrictor action of nicotine in the rabbit ear vessels. When a larger dose of nicotine was given which produced a large constriction, the smaller doses then lost all effect. The observation is consistent with the constrictor action of nicotine being due to release of noradrenaline taken up by the vessels from the perfusing fluid. After the large dose constriction was no longer seen because all had been released.

We often observed that an infusion of noradrenaline restored the action of tyramine to a greater extent than it restored the effect of sympathetic stimulation. A difference exists between them since the effect of sympathetic stimulation is blocked by bretylium whereas the action of tyramine is not. Tyramine may compete by mass action for the sites to which noradrenaline is attached, while sympathetic impulses may act differently. If noradrenaline is taken up into a store, tyramine may release it before it has reached the sites from which it is released by sympathetic stimulation.

Do the precursors restore the action of tyramine themselves, or must they first be converted to noradrenaline ? It is difficult to see how phenylalanine can act directly, and some conversion in the direction of noradrenaline seems more likely. If so, it is evidence that the conversion can occur very quickly.

The evidence that noradrenaline restored the pressor action of tyramine much more effectively than adrenaline was clear ; however, an infusion of adrenaline was found to restore the action of sympathetic stimulation in causing pupil dilatation, and an infusion of adrenaline seemed to be as effective as one of noradrenaline in increasing the inhibitory action of hypogastric stimulation on the uterus. A sharp distinction between noradrenaline and adrenaline in relation to the store at sympathetic nerve endings therefore cannot be made.

We wish to thank Miss Roneen Hobbs for the experiments in which rabbit ears were perfused. We also wish to thank Dr. H. Blaschko for a supply of *m*-tyrosine. This work has been done during the tenure by one of us (M. J. R.) of a Fellowship from the Life Insurance Medical Research Fund of Australia and New Zealand.

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SYMPATHETIC POSTGANGLIONIC CHOLINERGIC FIBRES

BY

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When the postganglionic fibres to the nictitating membrane are stimulated in a cat treated with reserpine, the membrane contracts. The contraction is increased after the injection of eserine and is abolished with atropine. Thus the fibres stimulated appear to be cholinergic. When the splenic nerve is stimulated in a cat treated with reserpine there is similar evidence of the presence of cholinergic fibres; the spleen dilates, the dilatation is greater in the presence of eserine and is abolished by atropine. The hypogastric nerves when stimulated cause inhibition of the uterus of the virgin cat. When the cat is treated with reserpine, stimulation then causes contraction which is increased in the presence of eserine and abolished by atropine. There is also evidence of cholinergic fibres in the postganglionic supply to the vessels of the rabbit's ear. From this and other evidence it is suggested that a form of adrenergic mechanism may exist such that cholinergic fibres in the postganglionic sympathetic supply liberate acetylcholine; this in turn liberates noradrenaline from a store around the sympathetic nerve ending.

Many effects produced in the body by stimulation of sympathetic nerves are also produced by acetylcholine (after atropine) and by nicotine acting peripherally. Vasoconstriction in the vessels of the isolated and perfused rabbit ear is an example. Since these effects of acetylcholine and of nicotine disappear (a) after treatment of the animal with reserpine and (b) after degeneration of the sympathetic nerves, it seemed that the effects might actually be mediated through some part of the sympathetic mechanism. Cholinergic fibres have been shown to occur in various sympathetic nerves, the first demonstration of them having been made by Euler and Gaddum (1931).

We have therefore carried out an investigation to see if fibres liberating acetylcholine were present in postganglionic sympathetic nerves generally, and have studied the supply to the nictitating membrane, the spleen, the uterus of the non-pregnant cat and the perfused rabbit ear. Bacq and Fredericq (1935) had already given evidence for the presence of cholinergic fibres in the nictitating membrane. We thought that if sympathetic nerves in general contained cholinergic fibres, the acetylcholine liberated might then have the same effect as injected acetylcholine or nicotine.

METHODS

Our method has been to study the effect of stimulating postganglionic sympathetic fibres in cats or rabbits treated with reserpine. This treatment reduces or removes the store of noradrenaline present around the sympathetic nerve endings and makes it easier to see effects which might be produced by release of acetylcholine.

Reserpine was dissolved in a 20% solution of ascorbic acid, and usually a 1% solution was prepared which was injected into the peritoneal cavity on two successive days. The animal was used for the experiment on the third day. The amount injected was 1.5 mg./kg. on each day. The animals were kept in a warm room at a temperature of 27°.

For observations on the nictitating membrane, the cat was anaesthetized with chloralose. The superior cervical ganglion was exposed on one side and a pair of electrodes was applied so that one was in contact with the ganglion and the other just beyond. The contractions of the nictitating membrane were recorded by an isotonic lever.

For observations on the spleen the cat was partially eviscerated from the colon to the middle of the duodenum, the inferior and superior mesenteric arteries being divided. The fibres of the splenic nerve were identified near the origin of the splenic artery and a ligature was tied around them. The fibres were not cut. A pair of shielded electrodes could then be placed so as to stimulate them. An incision was made in the skin on the left side and the muscle layers were separated by retraction with two aneurysm needles so that the spleen could be brought out of the body cavity and put into a plethysmograph (Burn, 1952). The volume changes were recorded by a piston recorder.

For observations on the uterus, the cat was eviscerated and the ligament between the kidney and the ovary was divided on one side. A thread was attached to the ovary and passed over pulleys to a

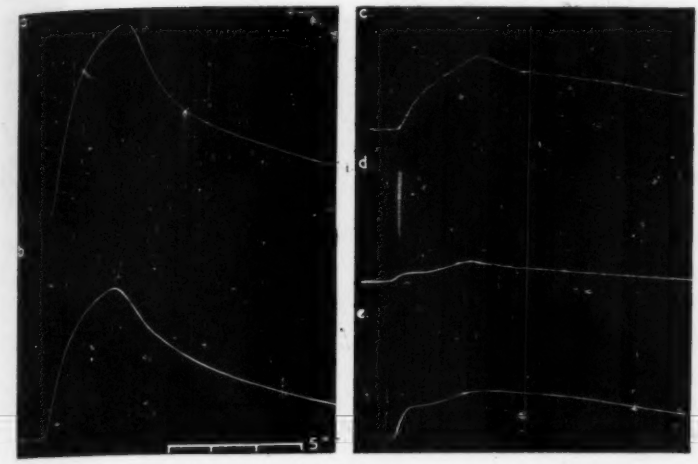


FIG. 1.—Contractions of nictitating membrane in response to postganglionic stimulation. (*a*) In normal cat, (*b*) in same cat after 4 mg./kg. atropine, (*c*) in cat treated with reserpine, (*d*) in same cat after 4 mg./kg. atropine, (*e*) in same cat after infusion of 0·5 mg. noradrenaline.

lever which recorded on the kymograph. The hypogastric nerves were identified and were passed through electrodes of a pattern recently described (Burn and Rand, 1960) which provided for a constant flow of oxygenated Krebs solution over the portion of nerve stimulated.

For observations on the perfused rabbit ear we used the preparation described by Gaddum and Kwiatkowski (1938) in which we applied electrodes to the fibres leaving the superior cervical ganglion. We also used the isolated ear, stimulating the mixed nerve at the base of the ear, using the perfusion system described by Burn (1952). For stimulation, the nerve was carefully dissected from its bed for about 7 mm. leaving both the central and the distal part undissected to keep the nerve in place. A pair of electrodes were placed around the dissected portion. Stimulation in all experiments was by square wave pulses usually of 2 msec. duration at a frequency of 25 per sec. The strength of stimulus was usually 1 mA. For observations on the pilomotor response to sympathetic stimulation in the cat's tail, cats were anaesthetized with chloralose and eviscerated to facilitate access to the lumbar sympathetic chain. We perfused the tail with Locke solution containing 4.5 g./l. sucrose at 35°, inserting the arterial cannula into the internal iliac artery of one side pointing towards the bifurcation; we tied the internal iliac of the other side and aorta just above the origin of the internal iliacs; thus the perfusing fluid passed into the caudal stump of the aorta. We stimulated the right lumbar sympathetic chain.

RESULTS

Nictitating Membrane.—In a previous paper (Burn and Rand, 1958b) we observed that when a cat had been treated with reserpine, although a large dose of tyramine failed to cause contraction of the nictitating membrane, stimulation of the postganglionic fibres was still able to do so. In Fig. 1 the effect of atropine on the contractions following sympathetic stimulation is shown. In a normal cat, the contraction was only slightly reduced by atropine even in the large dose of 4 mg./kg., but in a cat treated with reserpine it was very greatly reduced by this dose. In this cat the contraction increased after an intravenous infusion of 0.5 mg. noradrenaline.

Fig. 2 taken from an experiment in a cat treated with reserpine shows that eserine, given in two injections of 0.1 mg. each, augmented the contractions caused by sympathetic stimulation, especially those of weaker stimuli, and that atropine in a dose of 4 mg. (=1.5 mg./kg.) abolished the contractions. In two other experiments the effects of eserine and atropine were similar; in both, the effects of weaker stimuli were abolished by atropine and those of stronger stimuli greatly reduced. The threshold strength of stimulation was determined in three experiments and was found to be 0.39 mA., 0.075 mA.

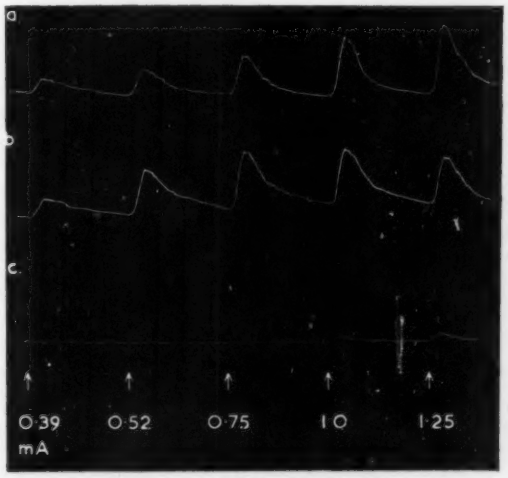


FIG. 2.—Contractions of nictitating membrane in response to postganglionic stimulation in cat treated with reserpine. (*a*) Responses to increasing strengths of stimulation as shown below, (*b*) increased responses after the injection of 0·2 mg. eserine, (*c*) responses abolished after the injection of 1·5 mg./kg. atropine.

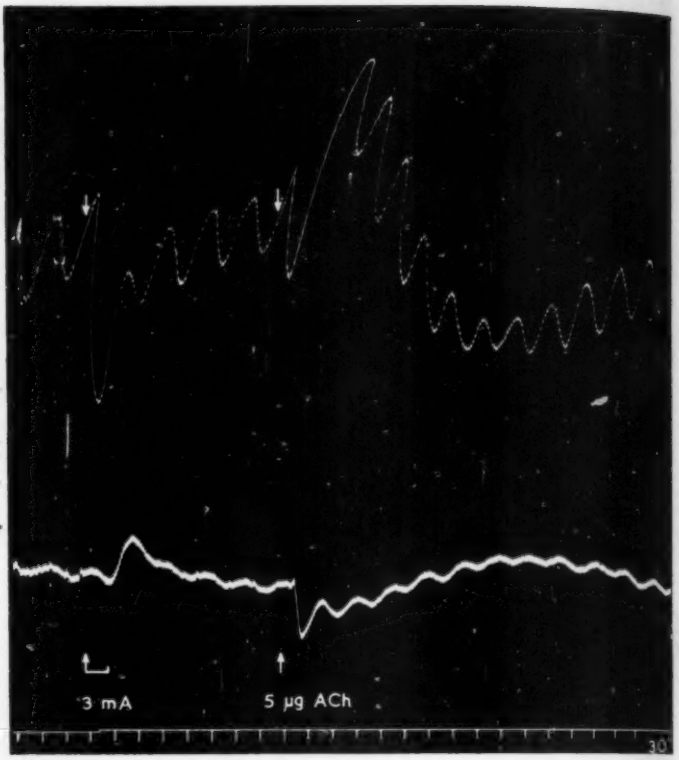
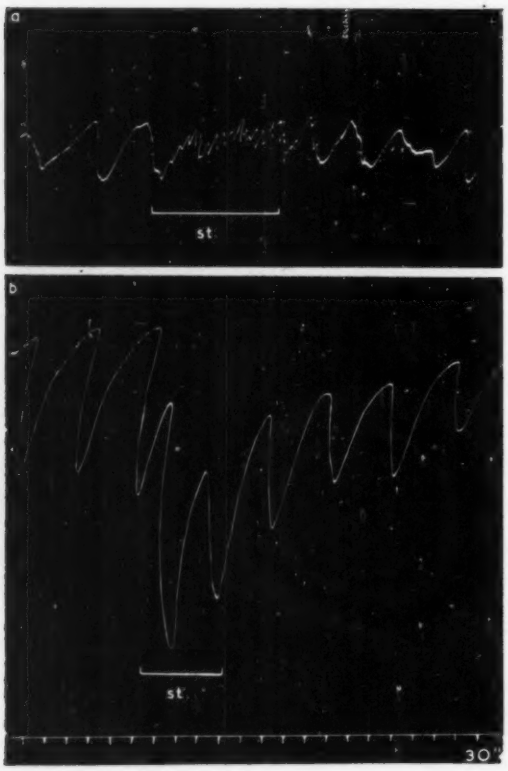


FIG. 3.—Spleen volume and blood pressure in cat treated with reserpine. Stimulation of splenic nerves for 30 sec. with strength 3 mA. caused small contraction of the spleen, and intravenous injection of 5 µg. acetylcholine caused dilatation followed by small slow contraction.

and 0.31 mA.; the threshold strength in one experiment on a normal cat was 0.25 mA.

Spleen.—Stimulation of the splenic nerve caused contraction of the spleen as shown in Fig. 3, in which the observation was made in a cat treated with reserpine. The effect was much smaller than that seen in a normal cat, but complete abolition of the contraction by reserpine treatment was rarely obtained. Fig. 3 also shows that in a cat treated with reserpine the intravenous injection of 5 µg. acetylcholine caused dilatation of the spleen, followed, as the blood pressure recovered, by a slight constriction. The stimulation of any cholinergic fibres which might be present in the splenic nerve would be expected to cause dilatation of the spleen, and would oppose the constrictor action of released noradrenaline.



Such a balance of opposed effects would explain the observations recorded in Fig. 4, also made in a cat treated with reserpine; at first stimulation caused no appreciable volume change but altered the record during the period of application, so that there were many small waves. Then 1 mg. atropine was injected, and 4 min. later stimulation was repeated and a constrictor action was recorded, as if the injection of atropine had excluded the dilator effect of acetylcholine which initially masked the constrictor effect.

In one experiment recorded in Fig. 5, in a cat which was treated with reserpine, the effect of sympathetic stimulation was to produce dilatation of the spleen. An injection of eserine was then given and a repetition of the stimulation caused a

FIG. 4.—Spleen volume of cat treated with reserpine. (*a*) Stimulation of splenic nerves in presence of eserine caused a series of small waves, but had no general effect on the volume. (*b*) The same stimulation after the injection of 1 mg. atropine caused contraction of the spleen.

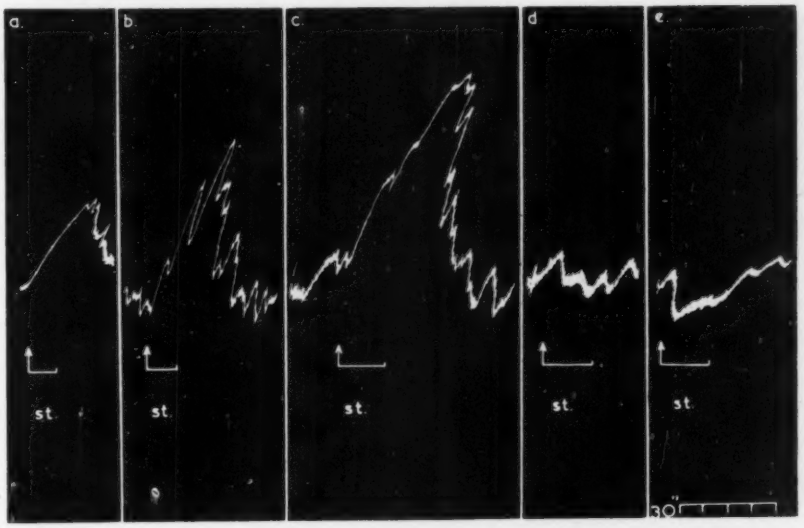


FIG. 5.—Spleen volume of cat treated with reserpine. (*a*) Stimulation of splenic nerves for 30 sec. caused dilatation of the spleen. (*b*) After the injection of 0·35 mg. eserine, stimulation for 30 sec. caused a greater dilatation. (*c*) Stimulation for 60 sec. caused still greater dilatation. (*d*) Stimulation for 60 sec. after 1·3 mg./kg. atropine had no effect. (*e*) Stimulation for 60 sec. after infusion of 0·25 mg. noradrenaline caused a very slight contraction.

rather larger dilatation. Stimulation was then applied for twice as long, causing a much larger dilatation. An injection of 4 mg. atropine was then given and 3 min. later stimulation had no effect. These were the principal observations, after which an infusion of 0.25 mg. noradrenaline was given. A further stimulation then caused a very slight constriction. An injection of tyramine which previously had no action caused a large constriction.

In other experiments of this kind in cats which were treated with reserpine, stimulation of the fibres of the splenic nerve caused a small constriction even in the presence of eserine ; it was, however, regularly observed that after the injection of 1 mg. atropine the constrictor response became 2 or 3 times greater than before.

Non-pregnant Cat Uterus. — Since the sympathetic innervation of the cat uterus when virgin or non-pregnant is inhibitory, observations were first made in normal cats and later in cats treated with reserpine. Fig. 6 is taken from an experiment in a normal virgin cat, and shows the inhibition due to stimulation of the hypogastric nerves. Eserine was then injected intravenously in three doses of 0.1 mg. at intervals of 5 min., and the stimulation was repeated. As a result the inhibition was diminished. Atropine was then injected in two doses each of 1 mg., and the stimulation was applied again ; the inhibitory effect was then greater.

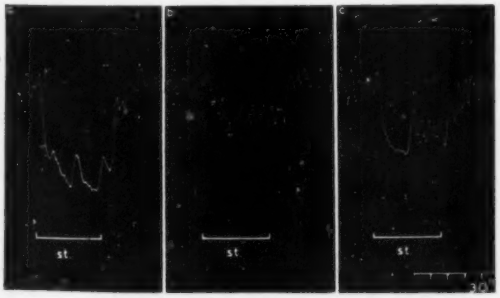


FIG. 6.—Uterus of virgin cat. (*a*) Stimulation of hypogastric nerves caused inhibition. (*b*) After injection of 0·3 mg. eserine the inhibition was reduced. (*c*) After the injection of 1 mg./kg. atropine the inhibition was increased.

In observations on virgin cats treated with reserpine, the inhibitory effect was absent as shown in Fig. 7. Stimulation of the hypogastric nerves caused a small contraction, which was augmented in the presence of eserine. Atropine was then injected, and stimulation no longer caused contraction, but a very slight relaxation. An intravenous injection of 0.5 μg. adrenaline caused relaxation. These results in the cat treated with reserpine were observed in 4 experiments.

An observation was also made on the isolated horn of the uterus of a normal virgin bitch ; it was found that acetylcholine caused contraction, but that in the presence of atropine it caused inhibition.

Perfused Rabbit Ear. — When perfusing the rabbit ear we did not find it easy to abolish the constrictor response to sympathetic stimulation by giving reserpine beforehand. Using the Gaddum-Kwiatkowski method we observed, however, that the effect of sympathetic stimulation was increased by adding eserine (5 μg./ml.) to the perfusing fluid. With Thorp's recorder it was possible to determine the volume of fluid (in drops) by which

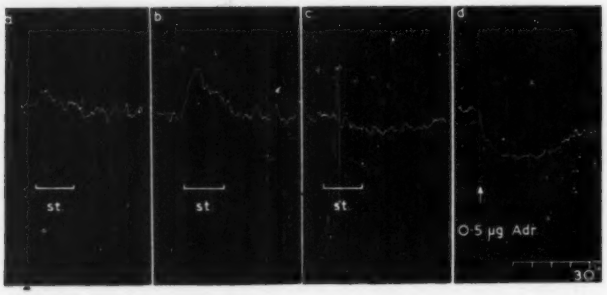


FIG. 7.—Uterus of virgin cat treated with reserpine. (*a*) Stimulation of hypogastric nerves caused slight contraction. (*b*) After injection of 0·3 mg. eserine, stimulation caused a greater contraction. (*c*) After the injection of 1 mg. atropine, stimulation caused a very slight relaxation. (*d*) Injection of 0·5 µg. adrenaline caused relaxation.

sympathetic stimulation, for a given time, reduced the outflow. Thus in one experiment the outflow was reduced by 330 drops in a first period of stimulation and by 356 drops in a second. Eserine was then added to the perfusion fluid and sympathetic stimulation then reduced the outflow by 486 drops. Similar observations were made in the isolated ear perfused through a cannula tied in the central artery at the base of the pinna. Fig. 8 shows the fall in outflow during stimulation of the mixed nerve, which was much greater after the addition of 3 µg./ml. eserine to the perfusion fluid.

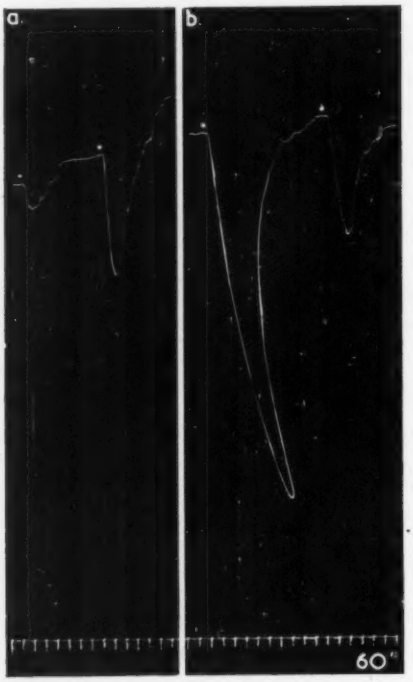


FIG. 8.—Record of outflow from rabbit ear during perfusion with Locke solution. (*a*) Stimulation of mixed nerve at the base of the ear for 10 sec. and then for 20 sec. caused slight reductions in outflow (5 mm. and 24 mm. on drum). (*b*) Stimulation 15 min. after eserine (2 µg./ml.) was added to the perfusion fluid, the duration of stimulation being respectively 20 sec. and 10 sec., caused larger reductions in outflow (75 mm. and 25 mm. on drum).

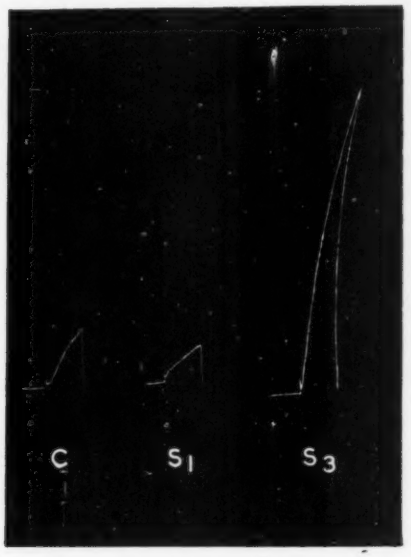


FIG. 9.—Tests of venous effluent from rabbit ear vessels on eserinized leech muscle. Perfusion with Locke solution containing eserine 5 µg./ml. At (C), effect of effluent before stimulation. At (S_1) effect of effluent during stimulation for 1 min. At (S_3) effect of effluent collected during the third min. after stimulation.

Using the Gaddum-Kwiatkowski perfusion we collected the venous effluent and tested it on the leech. Fig. 9 shows that samples collected after stimulation had a stimulant action on the leech muscle. Similar positive results were obtained in 5 experiments.

Block at Postganglionic Terminations. — We confirmed the observation of Brücke (1935) that, when a large amount of acetylcholine or of nicotine was injected into the skin at the base of a tuft of hair of the cat's tail, the effect of stimulation of the lumbar sympathetic chain was blocked, so that, while the adjacent tufts, respectively nearer to and further from the base of the tail, were erected during stimulation, the tuft at the site of injection remained unaffected. Fig. 10 gives an example of this action. A similar picture for nicotine is shown in Fig. 11.

These clear results were not obtained in all experiments, for the injection of nicotine or of acetylcholine not only blocked the effect of sympathetic stimulation, but partially blocked the effect of noradrenaline or adrenaline.

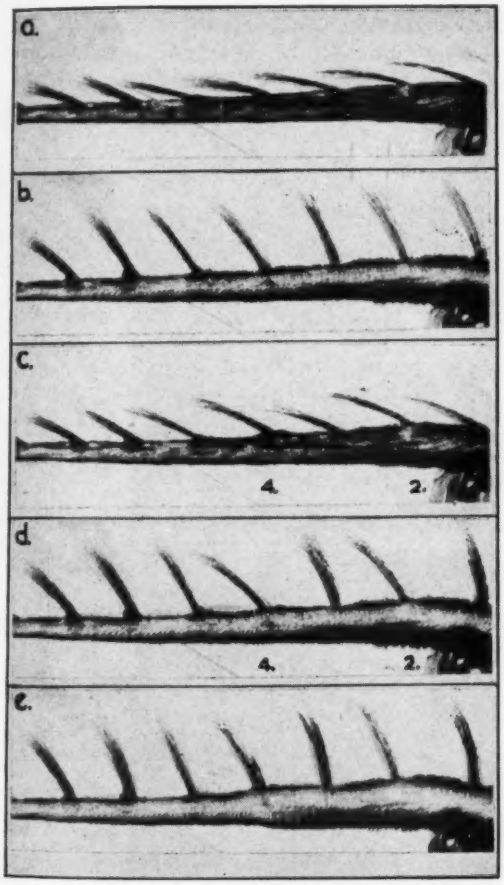


FIG. 10.—Pilomotor response in the tail of the cat (*a*) before stimulation, (*b*) during stimulation of right lumbar sympathetic chain, (*c*) after the intradermal injection of 0·2 mg. acetylcholine at the base of tufts 2 and 4, then (*d*) during stimulation tufts 2 and 4 were not fully erected, (*e*) after intravenous injection of 20 μg. noradrenaline all tufts were erected.

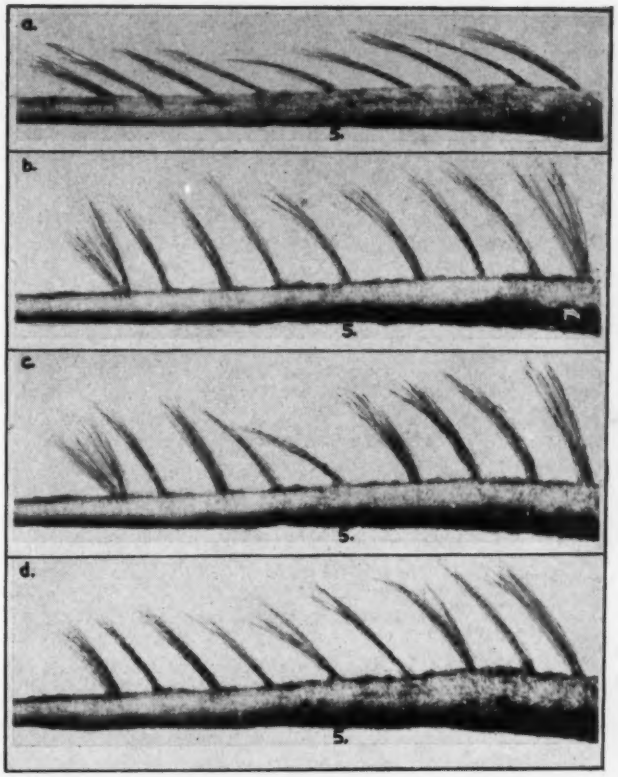


FIG. 11.—Pilomotor response in the tail of the cat. (*a*) Normal, (*b*) during sympathetic stimulation, (*c*) sympathetic stimulation, after intradermal injection of 0·2 mg. nicotine (base) at root of tuft 5, failed to erect this tuft, (*d*) intravenous injection of 30 μg. adrenaline caused erection of all tufts.

Other observations were made in the rabbit ear perfused with Locke solution by the method of Gaddum and Kwiatkowski (1938). We stimulated the postganglionic fibres and observed constriction. We observed that when acetylcholine was added to the Locke solution perfusing the ear, in concentrations from 2.5 to 20 μg./ml., the effect of sympathetic stimulation was blocked, but at the same time the constrictor effect of an injection of noradrenaline was reduced. Fig. 12 produces an illustration of this effect.

Observations with Choline 2,6-Xylyl Ether Bromide.—We had more success in blocking sympathetic effects with choline 2,6-xylyl ether bromide synthesized by Hey and Willey (1954) and investigated as a sympathetic blocking agent by Exley (1957). We tested its action by stimulation of the postganglionic fibres from the superior cervical ganglion both on the iris

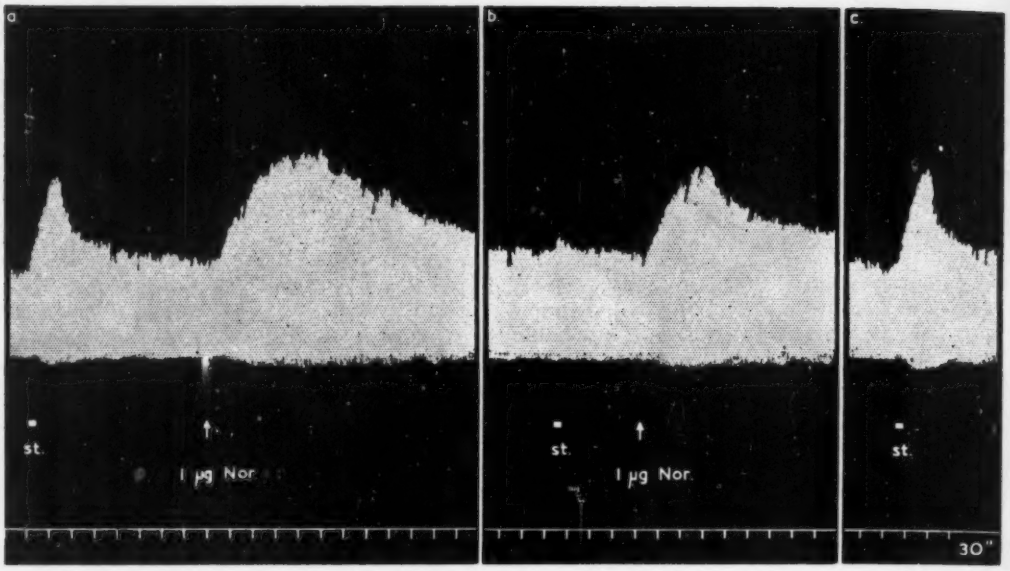


FIG. 12.—Gaddum drop-timer record of outflow from perfused rabbit ear. (*a*) At (st.): stimulation of postganglionic fibres caused constriction; at (1 µg. Nor): injection of noradrenaline. (*b*) Observations when 2·5 µg./ml. acetylcholine was added to perfusion fluid. At (st.): stimulation was blocked; at (1 µg. Nor): effect of injected noradrenaline was reduced. (*c*) At (st.): stimulation again effective when the ear was perfused without acetylcholine.

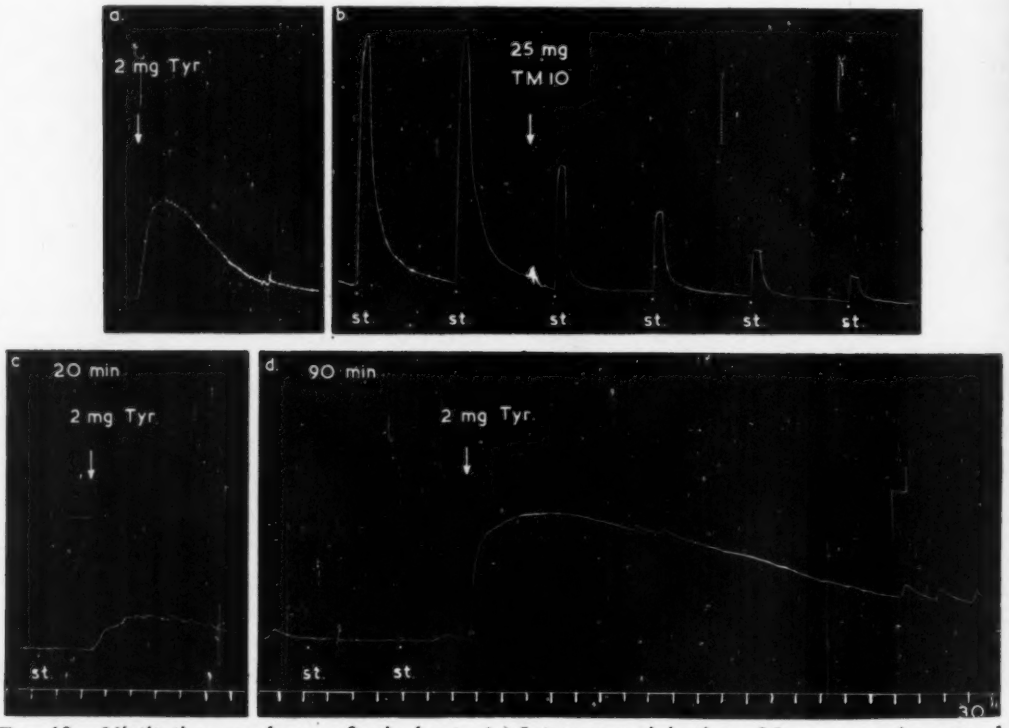


FIG. 13.—Nictitating membrane of spinal cat. (*a*) Intravenous injection of 2 mg. tyramine caused contraction, (*b*) stimulation of postganglionic fibres at 2 min. intervals. Injection of 25 mg. choline 2,6-xylyl ether bromide (TM10) reduced the response, (*c*) 20 min. after the injection of choline 2,6-xylyl ether bromide, the effect of stimulation was absent, and the effect of 2 mg. tyramine (Tyr) was reduced, (*d*) 90 min. after the choline 2,6-xylyl ether bromide, stimulation was ineffective even with a 6 times stronger stimulus, and 2 mg. tyramine had a greater effect than in (*a*).

and on the nictitating membrane. After the injection of 10 mg./kg. stimulation soon failed to act in both organs. Fig. 13 shows an experiment on the nictitating membrane in which the response to sympathetic stimulation was compared with that to tyramine. The immediate effect of choline 2,6-xylyl ether bromide was to depress the action of tyramine, but at the end of 90 min., when sympathetic stimulation with a strong stimulus was ineffective, the injection of tyramine produced a greater response than at first. Likewise tyramine still caused dilatation of the pupil. We also observed that the action of bretylium, N - *o* - bromobenzyl - N - ethyl - NN-dimethylammonium *p*-toluene-sulphonate (Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim, 1959), was essentially similar to choline 2,6-xylyl ether bromide in these respects, as shown in Fig. 14.

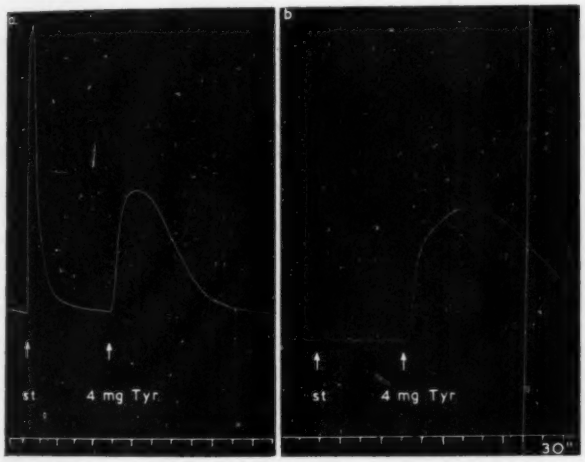


FIG. 14.—Nictitating membrane of spinal cat, 2·5 kg. (*a*) Contractions due to postganglionic stimulation (st.) and to 4 mg. tyramine (Tyr.), (*b*) 45 min. after injection of 25 mg. bretylium; stimulation ineffective, injection of 4 mg. tyramine had a greater effect than in (*a*).

Our colleague Huković also tested the action of choline 2,6-xylyl ether bromide and of bretylium on the constrictor action of acetylcholine observed in the presence of atropine in the perfused rabbit ear. He found that the constrictor response to acetylcholine and that to sympathetic stimulation was abolished, while that to tyramine was greater than before. Both substances also abolished the accelerator action of acetylcholine observed in the presence of atropine, and of sympathetic stimulation in the isolated atria, but augmented the action of tyramine.

DISCUSSION

Our previous observations (Burn and Rand, 1958b, 1960) have shown that the noradrenaline which can be extracted from tissues innervated by sympathetic fibres appears to play an important part in the action of tyramine and in the effect of sympathetic stimulation. The evidence was obtained from animals given reserpine and from normal animals. If the animal was treated with reserpine the extractable noradrenaline disappeared from the vessels, and both tyramine and sympathetic stimulation were then without effect. If an intravenous infusion of noradrenaline was given, both tyramine and sympathetic stimulation had an increased action, so that the effect of the infusion did not depend on the presence of reserpine. We have supposed that the infusion of noradrenaline partly refilled the store of extractable noradrenaline or in normal animals increased it. This is, however, a supposition, for attempts to demonstrate an increase of extractable noradrenaline have not succeeded as yet (Euler, 1956b).

Now acetylcholine and nicotine in the presence of atropine have been shown to exert a variety of actions which resemble those of stimulation of the sympathetic nerves. Since they lose these actions in animals treated with reserpine they may be acting by liberating noradrenaline from the store. These actions of acetylcholine and of nicotine, which are peripheral, include (a) vasoconstriction of the vessels of the rabbit ear (Burn and Rand, 1958a), (b) acceleration of the atria of the rabbit heart (Burn and Rand, 1958c), (c) pilomotor action in the cat's tail, (d) contraction of the nictitating membrane of the cat's eye (Burn, Leach, Rand and Thompson, 1959), and (e) inhibition of the isolated uterus of the virgin bitch. Moreover, de Burgh Daly and Scott (unpublished) have observed that acetylcholine injected into the splenic artery caused contraction of the spleen. We have found that intravenous injection of acetylcholine in the cat treated with reserpine caused dilatation of the spleen. There are in addition other actions which have not yet been demonstrated to be absent in the animal treated with reserpine. Ambache and Edwards (1951) observed that nicotine in the presence of atropine caused inhibition of the isolated ileum of the kitten.

The actions of acetylcholine and nicotine not only resemble those of the sympathetic nerves, but are in some way related to them, since the actions disappear after degeneration of the sympathetic

nerves. The constrictor action of nicotine in the perfused vessels of the rabbit ear, the pilomotor effect of nicotine and acetylcholine in the cat's tail, the contraction by nicotine of the nictitating membrane of the cat's eye, the contraction of the spleen by acetylcholine injected intra-arterially, are all lost after degeneration of the sympathetic nerves. This suggests that acetylcholine and nicotine act on part of the normal sympathetic mechanism, and it adds additional support to the suggestion that they release noradrenaline from the store near the nerve endings, since the store disappears when the sympathetic fibres degenerate (Euler and Purkhold, 1951 ; Burn and Rand, 1959).

If the store is part of the normal sympathetic mechanism and if acetylcholine can discharge noradrenaline from the store, the situation would become clarified if the postganglionic sympathetic fibres sending impulses to the store liberated acetylcholine.

Cholinergic fibres in sympathetic nerves were first demonstrated by v. Euler and Gaddum (1931) in the tongue of the dog. The classical case of a cholinergic innervation is, however, that in the sweat glands (Dale and Feldberg, 1935). Cholinergic fibres were then demonstrated in the sympathetic innervation of the vessels of the dog's hindleg (Bülbring and Burn, 1935) and in the innervation of the nictitating membrane (Bacq and Fredericq, 1935). Gaddum and Kwiatkowski (1939) observed that, in perfusions of the rabbit ear, stimulation of the great auricular and posterior auricular nerves liberated a substance in the venous effluent which caused contraction of the leech. However, they concluded that the liberation was not connected with sympathetic fibres, since the superior cervical ganglion had been removed two weeks before.

Recently Gillespie and Mackenna (1959) have found that, if rabbits were treated with reserpine, the isolated ileum was no longer inhibited by stimulation of the sympathetic fibres, but was caused to contract. The contraction was abolished by atropine, and Gillespie and Mackenna concluded that cholinergic fibres were present in the sympathetic supply. Recently also Huković (1959), working in this department, made a preparation of rabbit atria with sympathetic fibres attached. Stimulation of the fibres in the preparation from a normal rabbit caused acceleration of the atrial rate, but stimulation in a preparation from a rabbit treated with reserpine often caused inhibition, which was increased by eserine and abolished by atropine. He concluded that cholinergic fibres were present in the sympathetic supply.

In the present paper we have made similar observations in other tissues. Thus, in cats treated with reserpine, tyramine loses its action on the nictitating membrane altogether, but sympathetic stimulation does not ; it still causes a contraction. This contraction, however, we have found is abolished by atropine. When it has been abolished or greatly reduced by atropine it can be augmented again by an infusion of noradrenaline. This suggests that the fibres which are active in stimulating the nictitating membrane in the reserpine-treated animal are cholinergic. Similarly cholinergic fibres may be present in the splenic nerve, for, in some experiments from reserpine-treated animals, stimulation of these fibres caused dilatation of the spleen, like the injection of acetylcholine. In the nictitating membrane the effects of acetylcholine released from cholinergic fibres and of noradrenaline would appear to be additive. In the spleen the effects seem to be opposed, so that in reserpine-treated animals in which stimulation of the splenic nerve still caused contraction, the contraction was greatly increased after the administration of atropine, presumably because the dilator action of acetylcholine was abolished. Cholinergic fibres also seem to be present in the sympathetic supply to the virgin cat uterus. Here again the effects of acetylcholine and those of noradrenaline (or adrenaline) appeared to be opposed ; acetylcholine causes contraction of the virgin uterus, and stimulation of the hypogastric nerves in the reserpine-treated cat was found to cause contraction also, the contraction being increased by eserine and abolished by atropine. Thus a normally inhibitory sympathetic nerve supply seems to contain cholinergic fibres. Koelle (1955) from histochemical studies found that "there is probably some representation of typical cholinergic fibres in practically all predominantly adrenergic and sensory nerves." He went on to ask whether the terms cholinergic and adrenergic might refer to the predominant rather than to the exclusive types of transmitting agents of the nerve fibres.

Three observations respecting the postganglionic mechanism may now be considered together. First that the effect of sympathetic stimulation can be increased by infusing noradrenaline into the blood. Second that acetylcholine (after atropine), by a nicotine-like action exerted at the periphery, can reproduce sympathetic effects, and that it does so by causing a release of noradrenaline from the store at the nerve endings since it no longer acts when the sympathetic fibres have degenerated, or in the animal treated with reserpine. Third that

cholinergic fibres seem to be present wherever they are looked for in sympathetic fibres.

These observations at once suggest a function for the cholinergic fibres present in the sympathetic supply to organs like the spleen. The cholinergic fibres are probably adrenergic *in effect*, since the acetylcholine they discharge will act on the store of noradrenaline and liberate noradrenaline from it. This idea serves to explain an observation previously made by one of us (Burn, 1932) and illustrated in *J. Physiol.* (vol. 75, p. 150). There has hitherto been no explanation for it. When the lumbar sympathetic chain was stimulated during perfusion of the hindleg of a dog, a stimulus of 3 sec. duration caused a fall in arterial resistance. When the same stimulus was applied for 30 sec. the main effect was a rise in arterial resistance. It appeared that in stimulating for 3 sec. vasodilator fibres were excited, while in stimulating for 30 sec. vasoconstrictor fibres were excited. It may now be suggested that the 3 sec. stimulation resulted in a release of acetylcholine and a fall of arterial resistance due to the direct action of this substance; the 30 sec. stimulation resulted in a more prolonged release of acetylcholine, which was enough to liberate noradrenaline from the store. The usual conception of adrenergic fibres is that they are fibres directly liberating noradrenaline. We now have a second conception, namely, that fibres liberating acetylcholine may, by doing so, discharge noradrenaline from the store.

The cholinergic fibres may be blocked by excessive amounts of acetylcholine, as Brücke (1935) first showed in the pilomotor muscles of the cat's tail, and as we have seen in the vessels of the rabbit ear. The action of the blocking agents choline 2,6-xylyl ether bromide and of bretylium may be similar. These substances block sympathetic impulses completely, but do not prevent the action of tyramine. Indeed, 1 hr. after giving choline 2,6-xylyl ether bromide or bretylium, the action of tyramine is increased. Choline 2,6-xylyl ether bromide and bretylium, which block not only sympathetic stimulation but also the action of acetylcholine in producing sympathetic effects, seem well suited from their structure to prevent the action of sympathetic fibres from releasing noradrenaline from the store.

At the present time we have nothing more than hints on the site of the store and we mention them only to indicate directions in which further research might be made. v. Euler (1956) has expressed the view that the noradrenaline present in the spleen is probably within the fine terminations of the nerves since there is no evidence that it is located outside the nervous tissue itself. He calculated that the amount present would be from 3 to 30 mg./g., though the amount in the nerves before they reach the spleen is (in the ox) about 15 μg./g. If the store is located as v. Euler suggests, then the fine nerve terminals must be able to take up noradrenaline from the circulating blood. The fact that the store disappears when the nerves degenerate is consistent with the view that the store is within the nerves. On the other hand the fact that acetylcholine injected into the splenic artery can release noradrenaline from the store might indicate that the store is outside the nerves. It is conceivable that the store is held in chromaffin cells which have been described by Nordenstam and Adams-Ray (1957) and confirmed by Burch and Phillips (1958) in human skin, and have been observed by Leach (see Burn and Rand, 1958a) in the skin of the rabbit ear. They have also been observed in the cat's nictitating membrane and in the skin of the cat's tail (Burn, Leach, Rand, and Thompson, 1959). These cells disappeared in animals treated with reserpine, and in the nictitating membrane they also disappeared after degeneration of the sympathetic fibres. Much further work is, however, required to connect them with the store.

Our observations raise questions about the postganglionic sympathetic fibres, though they cannot be answered at present. Since the evidence for purely adrenergic fibres is very strong [obtained from histochemical examination of sympathetic ganglia (Koelle and Koelle, 1959) as well as from chemical identification of noradrenaline, dopamine and dopa decarboxylase in the fibres (Schümann, 1958)], there appear to be two mechanisms by which noradrenaline is released. The first is by simple release from the adrenergic fibres, and the second by the action of cholinergic fibres releasing noradrenaline from the store. These mechanisms seem fundamentally different, and it is, therefore, surprising that they should exist side by side; it is further surprising that both should be blocked by choline 2,6-xylyl ether bromide and by bretyllium.

Another question concerns the cholinergic fibres. Do some of these liberate acetylcholine to act directly, and others liberate it to set free noradrenaline from a store? The sweat glands are stimulated by the release of acetylcholine, but the cholinergic fibres to the muscle vessels of the dog's hindleg may certainly prove to be fibres whose main function is to release noradrenaline from a store, since in many dogs vasodilator

effects of stimulation are not seen unless special steps (such as the use of eserine) are taken to unmask them.

A conception which might unify the observations is that sympathetic postganglionic fibres were, like the other peripheral efferent fibres, originally cholinergic. In the course of evolution, for the purpose of developing an adrenergic mechanism, they came to innervate a store of noradrenaline, perhaps present in chromaffin tissue, so that the innervation in some respects resembled that of the adrenal medulla. In further development, the fibres were modified so that the noradrenaline in the chromaffin tissue was developed in the fibre itself, the true adrenergic fibres thus being formed. The blocking action of bretylium may, however, indicate that even these still possess some kind of cholinergic background. Thus postganglionic fibres may contain representatives of the three stages of development, the proportion varying according to the tissue innervated and perhaps according to the species.

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ANOXIA AND VENTRICULAR FIBRILLATION; WITH A SUMMARY OF EVIDENCE ON THE CAUSE OF FIBRILLATION

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Experiments are described showing that ventricular fibrillation is more readily produced in isolated rabbit hearts by electrical stimulation when the oxygen supply to the heart is reduced. This evidence completes investigations which have been made into factors affecting the production of fibrillation. These investigations have shown that factors which shorten the duration of the action potential, particularly those which cause the "plateau" to disappear, facilitate fibrillation, and those which lengthen the duration of the action potential prevent fibrillation. The reason for the length of the cardiac action potential may therefore be to prevent fibrillation. When the action potential is of normal length the fact that two adjacent fibres are out of phase does not matter; the one which is first to contract is not re-excited by the one which is second to contract, because at that moment the first is inexcitable. If the action potential is short, then the first may be already repolarized and may be excited by spread of excitation from the second. Factors which inhibit metabolism shorten the action potential.

When electrodes are attached to the ventricle of the isolated rabbit heart perfused through the aorta, a high rate of stimulation can then be applied for a given time. This causes the ventricles to fibrillate, as may be seen when the stimulation stops by using the electrodes as leads to an ECG. Ordinarily the fibrillation induced in this way continues only for 1 to 2 min. and a normal rhythm returns. However, under various conditions, such as in the presence of a low concentration of potassium or a high concentration of calcium, or in the presence of metabolic inhibitors, the fibrillation has been found to persist. The observations now to be described have been made to test the effect of a partial lack of oxygen.

METHODS

Rabbits were stunned and bled. The hearts were removed and attached to the apparatus for perfusion as already described (Armitage, Burn, and Gunning, 1957). The perfusion fluid contained (in mM.) NaCl 132, KCl 5.6, $CaCl_2$ 2.2, $NaHCO_3$ 2.5, $NaH_2PO_4.2H_2O$ 0.92, dextrose 11.1, sucrose 13.1. The solution was saturated with 95% oxygen+5% CO_2 for the control observations, and with 47.5% oxygen, 47.5% nitrogen and 5% CO_2 for the rest. The temperature of the perfusing solution was 32° and was maintained within ±0.5°, using the device of Saxby (1956). Hearts were perfused at a pressure of 50 cm. water. Platinum fish-hook electrodes were inserted in the wall of the left ventricle, one at the apex and the other near the base. The stimuli were 1–2 mA. in strength, 2 msec. in duration and applied at a frequency of 20/sec. for 3 min. When stimulation was not being applied, the electrodes were used as leads to a Cossor electrocardiograph (model 1314); a record of the rhythm was obtained every 5 min. during fibrillation. The solutions saturated with the two gas mixtures were kept in separate flasks and perfused from separate Marriotte bottles.

When perfusion with any solution was begun, it was continued for at least 15 min. before stimulation was applied. When after stopping stimulation a normal rhythm was restored within 5 min., the result was recorded as "no fibrillation." When fibrillation persisted for more than 5 min., the result was recorded as "fibrillation."

RESULTS

Effect of Anoxia.—The following is an example of an experiment to determine the effect of reducing the oxygen supply. An isolated rabbit heart was perfused with a solution saturated with 95% oxygen for 15 min. and was then stimulated electrically for 3 min. When the stimulation stopped the normal rhythm was resumed at once. The perfusion fluid was then changed to one

saturated with 47.5% oxygen and 47.5% nitrogen, and after allowing this solution to perfuse for 20 min., stimulation was applied again. When the stimulation stopped, the heart was fibrillating and continued to do so for the next 15 min. Ice-cold saline was then poured over the heart and a normal rhythm returned. The perfusion fluid was changed back to the solution saturated with 95% oxygen. Stimulation was applied for 3 min. and when it was stopped a normal rhythm returned at once. The perfusion fluid was again changed to one saturated with 47.5% oxygen and 47.5% nitrogen, and after 20 min. the stimulation was applied for 3 min. Again the heart fibrillated when the stimulation was turned off, and continued to do so for the next 15 min.

This straightforward result was not obtained in all experiments, for in some the heart did not fibrillate when the solution contained only 47.5% oxygen. Use was then made of the observation (Armitage, Burn, and Gunning, 1957) that, when the potassium concentration was lowered, the proportion of hearts fibrillating was increased. The following experiment is an example.

The heart was first perfused with the usual solution containing 5.6 mM. potassium. When the perfusion fluid was saturated with 95% oxygen, stimulation did not cause fibrillation. When the perfusion fluid was changed to one saturated with 47.5% oxygen, stimulation caused fibrillation, but this continued for 2 min. only and then a normal rhythm returned. The heart was next perfused with a solution containing a lower potassium concentration, 4.5 mM., and saturated with 95% oxygen. Stimulation caused fibrillation which continued for 1 min. only before a normal rhythm returned. The heart was then perfused with a solution also containing 4.5 mM. potassium but saturated with 47.5% oxygen. Stimulation then caused fibrillation which continued for more than 15 min.

In all, 14 experiments were carried out, and, in 9, evidence was obtained that the lower oxygen saturation caused the heart to fibrillate more readily. In 4 experiments the results were similar to those in the first experiment described, and were the more convincing because, after observing that when a heart was perfused with a solution saturated with 47.5% oxygen, stimulation caused persistent fibrillation, it was possible to return to the solution saturated with 95% oxygen and show that stimulation caused only transient fibrillation. The results of the 9 experiments are set out in Table I.

In Expt. No. 1 of Table I the potassium concentration was 4.2 mM., and the result of the first trial of stimulation, when the perfusion fluid was saturated with 95% oxygen, was that there was no fibrillation. The result of the second trial when the fluid was saturated with 47.5% oxygen was that persistent fibrillation developed. The result of the third trial was the same as that of the first.

TABLE I

EXPERIMENTS INDICATING THAT REDUCTION OF OXYGEN PERCENTAGE FAVOURED VENTRICULAR FIBRILLATION

The % oxygen represents the percentage of oxygen in the gas mixture with which the perfusion fluid was saturated.

Expt. No.	KCl (mM.)	No Fibrillation		Fibrillation	
		Trial	% Oxygen	Trial	% Oxygen
1	4·2	1	95	2	47·5
		3	95		
2	5·6	1	95	2	47·5
		3	95	4	47·5
3	5·6	1	95	2	47·5
		3	95		
4	5·6	1	95	2	47·5
		3	95		
5	5·6	1	95	2	47·5
6	5·6	1	95		
	5·6	2	47·5		
	4·5	3	95	4	47·5
7	5·6	1	95	2	47·5
8	5·6	1	95	2	47·5
				3	95·0
9	5·6	1	95	2	47·5
				3	95·0

There were 5 inconclusive experiments of which the following example is given. The heart was perfused with a solution containing 5.6 mM. potassium and was saturated with 95% oxygen. After stimulation fibrillation was transient. The heart was then perfused with the same solution saturated with 47.5% oxygen. When stimulation was applied, fibrillation was again transient. Perfusion was then continued with a solution containing a lower potassium concentration (4.4 mM.) and saturated with 95% oxygen. After stimulation this time, fibrillation was persistent. At the end of 15 min., fibrillation was stopped by pouring ice-cold saline over the heart. This result suggested that the reduction in potassium concentration had been too great, and a higher concentration (5.0 mM.) was tried. However, the heart fibrillated persistently when perfused with this solution saturated with 95% oxygen. Even

when a return was made to the original concentration of 5.6 mM. potassium, the heart perfused with the solution saturated with 95% oxygen fibrillated persistently after stimulation. Finally the heart was perfused with a solution containing 7.0 mM. potassium, but the heart did not fibrillate persistently after stimulation whether the solution was saturated with 95% or with 47.5% oxygen. With the lower saturation, however, it fibrillated for 4 min. There were 4 other similar inconclusive experiments which appeared to be due to an increasing tendency towards fibrillation as the duration of an experiment increased.

Discussion

The observations described show that when the perfusion fluid was saturated with a gas mixture containing 47.5% oxygen instead of 95% oxygen, electrical stimulation of the ventricles more readily produced persistent fibrillation. Now oxygen lack has been shown to shorten the duration of the cardiac action potential. Trautwein, Gottstein, and Dudel (1954) found that, in cat papillary muscle stimulated at 140/min., the duration of the action potential was diminished by oxygen lack, the plateau being lost. This effect was reversed when oxygen was supplied. The work was confirmed in a later paper (Trautwein and Dudel, 1956). Webb and Hollander (1956) showed that the action potential of the rat atrium was diminished by anoxia ; after 4 min. the diminution was 13%, after 10 min. it was 47% and after 17 min. it was 81%.

The evidence that the factors which facilitate fibrillation are those which shorten the action potential, and conversely that those which prevent fibrillation are those which lengthen it, is now considerable. Acetylcholine shortens the atrial action potential (Hoffman and Suckling, 1953) and facilitates atrial fibrillation in the dog (Burn, Vaughan Williams, and Walker, 1955). Acetylcholine has practically no effect on the ventricular action potential (Hoffman and Suckling, 1953) and has no effect on ventricular fibrillation (Armitage, Burn, and Gunning, 1957). Adrenaline lengthens the atrial action potential in the rat (Webb and Hollander, 1956), and prevents acetylcholine from causing atrial fibrillation (Burn, Gunning, and Walker, 1957).

Various metabolic inhibitors have been shown to shorten the action potential. Thus Macfarlane and Meares (1955) have shown that dinitrophenol reduces the plateau of depolarization in the atrium and ventricle of the frog to about one-third of its normal length, and Kuschinsky, Lüllmann and Muscholl (1958) have shown that it reduces the duration of the action potential in the atrial muscle of the rat. Dinitrophenol was found to facilitate fibrillation in the rabbit ventricle (Armitage, Burn, and Gunning, 1957), and this effect was reversible. Another metabolic inhibitor is sodium azide, which Macfarlane (1956) found would reduce the duration of the action potential in the frog ventricle, and in keeping with this Goodford (1958) found that, in the presence of sodium azide, rapid stimulation of the ventricles led to persistent fibrillation as in the presence of dinitrophenol. The effect of sodium azide was reversible.

A third metabolic inhibitor is sodium monoiodoacetate. Lüllmann (1959) found that in its presence the duration of the action potential in the atria of the rat was shortened, and Goodford (1958) found that in its presence rapid stimulation of the ventricles caused fibrillation. This effect was not reversible.

A lack of glucose has also been shown to shorten the action potential in the rat atrium, the shortening becoming greater, the longer the time of glucose deprivation (Webb and Hollander, 1956). Goodford (1958) found that after perfusing rabbit hearts with glucose-free solution they continued to beat normally for periods of 2 hr. or more. By the end of that time some hearts began to fibrillate spontaneously, and, when the perfusion fluid was changed to one containing glucose, the fibrillation was arrested.

A factor which works in the opposite direction is a fall of temperature. The effect of lowering the temperature on the duration of the action potential was observed by Trautwein, Gottstein, and Dudel (1954) in cat papillary muscle. They found that in the range 38°–19°, the action potential, particularly the plateau, became longer as the temperature fell. The same observation was also made by Hollander and Webb (1955). Marshall (1957) observed the same change in the transmembrane potential of single fibres of the rabbit atrium ; the duration of the action potential rose from 240 msec. to 400 msec. as the temperature fell from 27° to 18°. Now fibrillation can be arrested by pouring ice-cold saline over an isolated heart (Dirken, Gevers, Heemstra and Huizing, 1955). Beaulnes and Day (1957) working on isolated atria found that fibrillation produced at 37° was arrested when the temperature was lowered to 29° and began again when the temperature was raised. Similar observations were made by Goodford (1958) on the ventricles ; he found that fibrillation

was arrested when the temperature was lowered from 37° to 32°.

Changes in calcium concentration affect the duration of the action potential. Hoffman and Suckling (1956) have found that the plateau was prolonged and the action potential was longer when the calcium concentration was low. When the calcium concentration was raised, repolarization occurred at a faster rate. Milton (1959) has shown that the proportion of hearts fibrillating in response to electrical stimulation is directly proportional to the calcium concentration in the perfusing fluid.

Changes in potassium concentration finally have a special interest, since there are two effects. Weidmann (1956) showed that large amounts of potassium shortened the action potential of the turtle ventricle. Vaughan Williams (1959) also found that in rabbit atria the action potential was shorter at 10 mM. concentration than at 5 mM. However, below 5 mM. the action potential also shortened as the concentration was reduced. Now, Milton (1959) found that when the potassium concentration was decreased below 5.6 mM. the proportion of hearts fibrillating increased. On the other hand, high concentrations of potassium are known to have a fibrillatory action. For example, Grumbach (1956) found that the injection of 1 ml. 0.77 M. potassium chloride into the fluid perfusing an isolated heart caused fibrillation if the heart had been exposed to adrenaline earlier.

Having thus seen several examples which point to a connexion between the duration of the action potential and fibrillation, we may ask what the connexion is. When the action potential in a fibre is short the fibre becomes re-excitable very soon, and, if some nearby fibres are contracting, a spread of the excitation from them may cause the fibre to contract again.

We ordinarily suppose that an impulse from a pacemaker arrives at distant fibres simultaneously. But, when the impulses come quickly, the rate of conduction from the pacemaker to two distant but adjacent points may not be identical, and the impulse may not arrive simultaneously at the two fibres. One will then contract before the other contracts. Provided the action potential is long, the first fibre will be inexcitable when the second contracts. But if the action potential is short, and the first fibre is rapidly repolarized, then this fibre will be stimulated to contract again by spread of excitation from the second fibre. There will be a local extrasystole.

Thus fibrillation could result from the spread of excitation from one fibre to another, when the fibres are out of phase and when the action potential is very short. The purpose of the long action potential of cardiac muscle seems to be to prevent this spread of excitation and so to prevent fibrillation. It is clear that the maintenance of the long action potential depends on a process which requires energy, since it is not maintained when there is lack of oxygen or glucose, or when metabolic inhibitors are present.

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THE NEUROMUSCULAR BLOCKING PROPERTIES OF A SERIES OF BIS-QUATERNARY TROPEÏNES

BY

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Linkage of two tropine esters through their nitrogen atoms by the chain $-[CH_2]_m-O-CO-[CH_2]_n-CO-O-[CH_2]_m-$, in which m was 2 or 3 and n varied from 0 to 6, gave compounds which produced neuromuscular block without depolarization. Reversibility by neostigmine was confirmed for a few compounds. Potency was found to depend upon the tropine ester employed and upon the values of n and m. Short duration and hypotensive properties were favoured by the higher values of n. The duration of action of the compound based on the phenylacetic acid ester of tropine, in which $n=4$ and $m=2$, varied considerably in different species. Epimerization, in which the relative positions of the methyl group and the linking chain on the quaternary tropane nitrogen atom were reversed, did not produce substances having more favourable properties than those possessed by the unepimerized compounds.

Bis-onium compounds in which the quaternary nitrogen atoms are present as part of substituted tropane nuclei have been examined for neuromuscular blocking properties by Gyermek and Nádor (1957). Some were considerably more active than tubocurarine in laboratory animals, but so far none has shown sufficient advantages over muscle relaxants currently in use to justify its introduction into clinical practice.

Linking of the tropane nuclei in such compounds has been effected in three ways; by means of an alkyl or aralkyl chain between the nitrogen atoms of the tropane nuclei (Kimura and Unna, 1950; Gyermek and Nádor, 1952; Eckfield, 1959), through the tropic acid hydroxyl groups of two atropine molecules with the formation of ether linkages (Kimura, Unna and Pfeiffer, 1949), or through the 3-hydroxyl groups of quaternized tropine molecules to form di-esters of dicarboxylic acids such as isatropic (Hotovy, Jacobi and Kuessner, 1956; Just, 1953), succinic or phthalic acid (Gyermek and Nádor, 1953).

The esterifying acid, the quaternizing group used, and the chain length are all known to be of importance in determining potency and side actions (Gyermek and Nádor, 1957; Haining, Johnston and Smith, unpublished observations), but the effect of chain structure on duration of action has received little attention. In $\alpha\omega$-dicarboxylic esters of choline the presence of ester groups at suitable points within the chain shortens duration of action (Brücke, 1956). However, potent members of this series, such as suxamethonium, have disadvantages (Churchill-Davidson, 1958), one of the most important being that, since they are depolarizing agents, paralysis due to them cannot be reversed by neostigmine. On the other hand, bis-quaternary tropeïne neuromuscular blocking compounds tend to exert a competitive type of action (Haining *et al.*, unpublished observations).

The compounds described here were prepared in the hope that, by linking two quaternary tropeïne moieties by a chain susceptible to attack by esterases, it would be possible to obtain competitive neuromuscular blocking agents of short duration.

METHODS

Neuromuscular Block in Cats, Dogs and Rabbits.—The neuromuscular blocking activity of compounds was investigated in cats anaesthetized with a mixture of chloralose 50 mg./kg. and urethane 500 mg./kg. given by intraperitoneal injection, or in dogs which had received pentobarbitone sodium intraperitoneally. One hind leg was fixed rigidly in a vertical position by means of a pin through the lower end of the femur. Shielded silver electrodes were placed on the sciatic nerve which was then crushed proximal to the electrodes. The gastrocnemius muscle was attached to a flat spring myograph and the contractions recorded on smoked paper. Muscle twitches were elicited by supramaximal rectangular pulses of less than 1 msec. duration.

The standard used throughout these experiments was *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(3-phenylacetoxytropanium bromide) (DF596). The relative potency of this compound in different species has been reported previously (Haining, Johnston and Smith, 1959). It was approximately half as potent as tubocurarine in the cat, and the recovery rate was approximately equal to that of suxamethonium (Fig. 1). DF596 has the advantage over compounds such as gallamine triethiodide or tubocurarine commonly used as standards in that it has little or no cumulative properties. This allowed considerably more estimates to be made in any given period of time. The quantities in the text refer to chloride unless otherwise stated. The potency and duration of action of compounds and their effects on blood pressure were determined by comparison with DF596 in the same animal. Whenever possible doses of each compound were chosen which would reduce the height of the gastrocnemius twitch by approximately 25% and 75%. The percentage reduction in twitch height and fall in blood pressure resulting from each dose were plotted against the logarithm of the dose. Since the slopes of the regression lines varied with the rate of recovery, potencies were compared at doses estimated to give a 50% reduction of twitch, and effects on blood pressure were compared at these doses. Estimates of duration of action were made by visual comparison of tracings. Values obtained for long-acting compounds were least reliable since, with compounds of the same duration as gallamine triethiodide, cumulative effects made comparisons difficult.

Blood pressure was recorded from the carotid artery with a mercury manometer. Animals were usually maintained with artificial respiration. Drugs were dissolved in physiological saline and were injected into the femoral vein.

In order to determine the rate of recovery in rabbits, drug solutions were infused into the marginal ear vein by means of a continuous slow injector (Palmer). Head drop was judged to be complete when the rabbit was unable to raise its head if tapped gently on the nose with a finger. The infusion was then stopped. Times required for the following stages in recovery were recorded: (1) The animal was able to raise its head in response to a light tap on the nose. (2) The animal was able to right itself immediately when quickly placed on its back. (3) With the hind limbs lifted clear of the ground, the animal was able to support itself and walk on its forelimbs. The time for complete recovery, when the animal was able to walk without assistance, was also determined. This, however, could not be obtained so precisely as with the other measurements, since animals after recovering from paralysis usually showed little inclination to move. During infusions, animals were allowed to sit on a rubber mat, and were restrained slightly with the hand. They usually remained quite still as long as they were able to get a good grip with their feet.

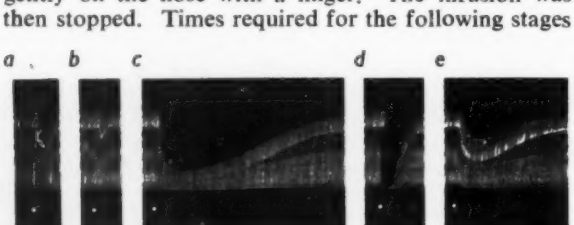


FIG. 1.—Cat. Maximal twitches of the gastrocnemius muscle elicited by indirect stimulation. Time, min. All drugs were given intravenously. (*a*) At time 0 min., 0.5 mg./kg. of DF596 was given. (*b*) At 7 min., 0.3 mg./kg. of DF596. (*c*) At 14 min. 0.3 mg./kg. of tubocurarine chloride. (*d*) At 67 min., 0.8 mg./kg. of DF596. (*e*) At 78 min., 0.15 mg./kg. of tubocurarine chloride.

The effects of large intravenous doses in conscious rabbits were investigated in animals maintained with the aid of a Drinker-type respirator. The rabbit was placed in a Perspex box with head protruding and chin resting on a support. A good seal at the neck was obtained with a collar of tambour rubber. Partial vacuum within the box was obtained by an Edwards two-stage vacuum pump 2SC50, and was broken at intervals by a simple spring-loaded flap valve. An adjustable leak consisting of a plate sliding over a slit in the box was provided to allow for various sizes of rabbits. Initially the valve was operated by a relay and timing circuit (Austin, 1954), but, as this was extremely noisy and gave jerky respiratory movements, it was found more convenient to operate the valve by means of a cam fitted on the shaft of a windscreen wiper motor (Lucas type C.W.1).

The Isolated Frog Rectus Abdominis Muscle Preparation.—Muscle strips were suspended in frog Ringer solution in a 10 ml. bath. Regular contractures to acetylcholine were obtained at 5 min. intervals. Antagonists were added to the bath 1 min. before the agonist, which was allowed to act for 1 min. When compounds were tested for ability to produce a contracture they were allowed to act for 5 min.

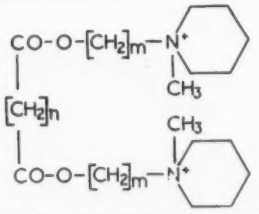
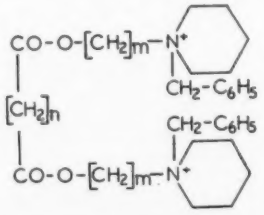
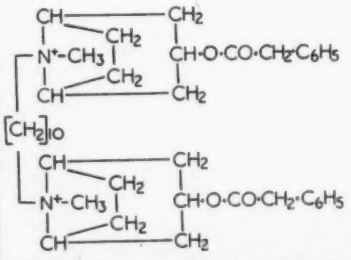
Action in Chicks.—To determine mode of action, compounds were administered subcutaneously to day-old chicks which were then observed until they died (Buttle and Zaimis, 1949). Drugs known to have competitive or depolarizing action were used at the same time for purposes of comparison.

The Isolated Human Foetal Phrenic Nerve-Diaphragm Preparation.—These preparations were obtained from foetuses of between 16 and 24 weeks. Sectors of diaphragm with nerve and ribs attached were removed from the foetus within 1 or 2 hr. of delivery, placed in Tyrode solution (NaCl, 0.9%; KCl, 0.042%; $CaCl_2$, 0.024%; $NaHCO_3$, 0.05%; dextrose, 0.1%) for transport to the laboratory. On arrival they were set up in an organ bath in oxygenated Tyrode solution at 36° to 37° within a further 30 min. Maximal contractions were obtained by stimulating the muscle indirectly with rectangular pulses of less than 1 msec. duration at a rate of about 5/min.

TABLE I

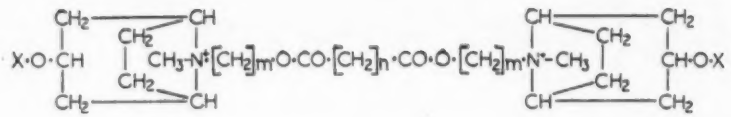
RELATIVE POTENCY, DURATION OF ACTION AND TYPE OF ACTION SHOWN BY BIS-QUATERNARY TROPEÏNE COMPOUNDS IN VARIOUS TESTS

DF596 was used as the standard for comparisons of potency and duration except for DF41. Col. 1, Relative potency in the cat. Number of estimates in parentheses. Col. 2, Relative duration of action in the cat. Col. 3, Degree of hypotension produced in the cat. Shown as $<$, $\equiv$ (equivalent to), $>$ that due to DF596. Col. 4, Anti-acetylcholine activity on the isolated guinea-pig ileum. Atropine sulphate = 1·0. Col. 5, Action on the frog rectus abdominis muscle. C, compound produced a contracture. A, compound antagonized the action of acetylcholine. Col. 6, Type of paralysis produced by injection into chicks. F, flaccid. S, spastic. † Indicates that the compound was also tested in rabbits (Table II). * Indicates that neuromuscular block was reversed by neostigmine. ‡ Comparison with tubocurarine.

Series	DF No.	Name of Compound	Structure	Anion	*m*	*n*	1	2	3	4	5	6	
		Suxamethonium chloride						++					†
		Tubocurarine chloride						+++++					†
		Gallamine triethiodide						++++ to +++++					†
	616	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(1-methylpiperidinium iodide)		2 I^-	2	4	0·6 (2)	+ to ++	$<$		C	S	
	628	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(1-benzylpiperidinium bromide)		2Br^-	2	4	$<$0·2 (1)				A	S F	
	41	*NN'*-Decamethylenebis(3-phenylacetoxytropanium bromide)		2Br^-			0·8 (2) ‡	++++ to +++++ ‡		0·02		F	† *

(Continued overleaf)

TABLE I—*continued*



			X	Anion	*m*	*n*	1	2	3	4	5	6	
I	623	*NN'*-4,7-Dioxo-3,8-dioxadecamethylenebis(3-acetoxytropanium bromide)	-CO-CH$_3$	2Br$^-$	2	2	0·2 (1)	++++	<		A		
	632	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(3-acetoxytropanium bromide)		2Br$^-$	2	4	0·25 (2)	+++	<		A		
	631	*NN'*-4,11-Dioxo-3,12-dioxatetradecamethylenebis(3-acetoxytropanium bromide)		2Br$^-$	2	6	0·4 (2)	++	≡		A		
II	619	*NN'*-4,6-Dioxo-3,7-dioxanonamethylenebis(3-benzoyloxytropanium bromide)	-CO-C$_6$H$_5$	2Br$^-$	2	1	0·5 (1)	++++			A	F	
	618	*NN'*-4,7-Dioxo-3,8-dioxadecamethylenebis(3-benzoyloxytropanium bromide)		2Br$^-$	2	2	0·7 (1)	++++	≡		A	F	†
	620	*NN'*-4,9-Dioxo-3,10-dioxadodecamethylenebis(3-benzoyloxytropanium bromide)		2Br$^-$	2	4	1·2 (2)	+++	<		A	F	†
	622	*NN'*-4,10-Dioxo-3,11-dioxatridecamethylenebis(3-benzoyloxytropanium bromide)		2Br$^-$	2	5	1·3 (3)	++	>		A		†
	634	*NN'*-4,11-Dioxo-3,12-dioxatetradecamethylenebis(3-benzoyloxytropanium bromide)		2Br$^-$	2	6	0·2 (2)	+	>		A		
III	604	*NN'*-4,5-Dioxo-3,6-dioxaoctamethylenebis(3-phenylacetoxytropanium bromide)	-CO-CH$_2$-C$_6$H$_5$	2Br$^-$	2	0	<0·2 (2)		>	0·01	A	F	
	595	*NN'*-4,6-Dioxo-3,7-dioxononamethylenebis(3-phenylacetoxytropanium bromide)		2Br$^-$	2	1	1·2 (3)	+++ to ++++	<	0·01	A	F	† *
	593	*NN'*-4,7-Dioxo-3,8-dioxadecamethylenebis(3-phenylacetoxytropanium bromide)		2Br$^-$	2	2	1·0 (1)	+++ to	<	0·01	A	F	†

		enebis(3-phenylacetoxytropanium bromide)	-CO-CH_2-C_6H_5											
III	593	*NN'*-4,7-Dioxo-3,8-dioxadecamethyl-		2Br⁻	2	2	1·0 (2) (1)	+++ to	<	0·01	A	F	†
		enebis(3-phenylacetoxytropanium bromide)												
	596	*NN'*-4,9-Dioxo-3,10-dioxadodeca-methylenebis(3-phenylacetoxy-tropanium bromide)		2Br⁻	2	4	1·0	++		0·1	A	F	† *
	621	*NN'*-4,10-Dioxo-3,11-dioxatrideca-methylenebis(3-phenylacetoxy-tropanium bromide)		2Br⁻	2	5	0·4 (2)	+	>		A	F	
	635	*NN'*-4,11-Dioxo-3,12-dioxatetradeca-methylenebis(3-phenylacetoxy-tropanium bromide)		2Br⁻	2	6	<0·1 (3)	+	>		A		
	696	*NN'*-4,9-Dioxo-3,10-dioxadodeca-methylenebis(3-β-phenylpropionyl-oxytropanium bromide)	-CO-CH_2-CH_2-C_6H_5	2Br⁻	2	4	0·9 (2)	++	< ≡		A		†
	767	*NN'*-5,7-Dioxo-4,8-dioxaundecamethyl-enebis(3-phenylacetoxytropanium bromide)		2Br⁻	3	1	1·6 (1)	+++	<		A		
	625	*NN'*-5,8-Dioxo-4,9-dioxadodecamethyl-enebis(3-phenylacetoxytropanium iodide)		2I⁻	3	2	1·8 (3)	+++	<		A		† *
IV	653	*NN'*-5,9-Dioxo-4,10-dioxatrideca-methylenebis(3-phenylacetoxy-tropanium iodide)	-CO-CH_2-C_6H_5	2I⁻	3	3	1·2 (2)	++	<		A		
	646	*NN'*-5,10-Dioxo-4,11-dioxatetra-decamethylenebis(3-phenylacetoxy-tropanium iodide)		2I⁻	3	4	0·6 (2)	+	≡		A		
	649	*NN'*-5,12-Dioxo-4,13-dioxahexamethyl-enebis(3-phenylacetoxytropanium iodide)		2I⁻	3	6	0·1 (2)	+	>		A		
	723	*NN'*-6,7-Dioxo-5,8-dioxadodecamethyl-enebis(3-phenylacetoxytropanium bromide)	-CO-CH_2-C_6H_5	2Br⁻	4	0	0·2 (1)	+++ to ++++	>		A		†
	686	Epimer of DF593		2Br⁻	2	2	0·5 (1)	+++ to ++++	<		A		†
V	668	Epimer of DF596	-CO-CH_2-C_6H_5	2Br⁻	2	4	0·8 (1)	+++			A		†
	719	Epimer of DF635		2Br⁻	2	6	0·2 (2)	+	>		A		

Anti-acetylcholine Activity.—This was determined on the isolated guinea-pig ileum preparation by the method of superfusion (Adam, Hardwick and Spencer, 1954). Regular submaximal contractions were obtained with acetylcholine at 90 sec. intervals. Antagonists were dissolved in Tyrode solution and applied to the gut for a period of 75 sec. before the application of acetylcholine. The effects of several concentrations of antagonist were compared with those of several of atropine sulphate. The percentage reduction in the height of contraction was plotted against the logarithm of the concentration. Since regression lines were not always parallel, comparisons of concentrations required to reduce the height of contractions by 50% were made.

Results

Table I shows the structure of compounds investigated and the results of tests carried out in cats in which their potency, duration and effect on blood pressure were compared with those of DF596. Relative activity in antagonizing the action of acetylcholine on the isolated guinea-pig ileum, the effect produced on frog rectus abdominis muscle, and the type of paralysis resulting from injection into chicks are also shown.

Table II gives the mean doses of the compounds necessary to cause head drop in rabbits, and the mean recovery times with standard deviations, in each case. The head-drop doses apply only to the particular drug concentrations and infusion rates employed, since with the short-acting compounds variations in either parameter resulted in a marked alteration of estimated potency.

Of the possible variations to the basic structure shown in Table I, the effects due to the following were investigated; changing the tropine ester, reversing the conformation of the methyl group and the linking chain about the nitrogen atom of the tropane nucleus, and altering the values of *n* and *m*.

Derivatives of Phenylacetyltropeïne

Compounds derived from phenylacetyltropeïne in which *m* was 2 (Series III) were investigated in greatest detail. To obtain neuromuscular blocking potency of the same order as DF596, the value of *n* required was not critical in the range 1 to 4. If the chain was lengthened or shortened, there was a drop in potency. In cats, the oxalic acid ester [DF604, *NN'*-4,5-dioxo-3,6-dioxaoctamethylenebis(3-phenylacetoxytropanium bromide)] had less than one sixth the activity of the malonate [DF595, *NN'*-4,6-dioxo-3,7-dioxanonamethylenebis(3-phenylacetoxytropanium bromide)] whilst the suberic acid ester [DF635, *NN'*-4,11-dioxo-3,12-dioxatetradecamethylenebis(3-phenylacetoxytropanium bromide)] was less than one tenth as effective as the adipate (DF596).

Table II

POTENCY AND DURATION OF ACTION OF BIS-QUATERNARY TROPEÏNE COMPOUNDS ESTIMATED BY THE HEAD DROP TEST IN RABBITS

Compound	Infusion Rate (ml./min.)	Conc. (mg./ml.)	Mean Head Drop Dose (mg./kg. with S.D.)	Revovery Time in Min. with S.D.			
				Head Drop	Righting Reflex	Fore Limbs	Walking Unaided
Suxamethonium chloride	1·0	0·3	0·32 (0·07)	2·7 (0·7)	3·9	7·2	7·5 (1·4)
Tubocurarine ,,	0·5	0·2	0·18 (0·04)	8·2 (3·3)	9·9	14·3	17·7 (4·0)
Gallamine triethiodide ..	0·5	0·5	0·43 (0·08)	5·5 (1·3)	7·4	10·4	12·2 (3·4)
DF41	0·5	0·2	0·24 (0·06)	3·6 (2·0)	4·1	4·9	5·4 (1·9)
DF618	0·5	0·3	0·33 (0·07)	1·7 (0·6)	2·3	3·0	3·4 (2·2)
DF620	0·5	0·3	0·21 (0·07)	1·9 (0·7)	2·3	3·2	3·9 (0·9)
DF622	1·0	0·3	0·23 (0·03)	2·9 (1·0)	3·6	5·0	5·3 (1·1)
DF595	0·5	0·3	0·29 (0·05)	2·1 (0·5)	2·2	3·0	3·4 (0·9)
DF593	0·5	0·3	0·32 (0·10)	2·0 (0·5)	2·0	3·2	3·6 (1·1)
DF769	0·5	0·3	0·15 (0·03)	2·5 (1·3)	2·8	3·9	4·6 (1·0)
DF596	1·0	0·3	0·30 (0·13)	1·5 (0·6)	2·2	2·7	3·6 (1·0)
DF696	1·0	0·3	0·33 (0·10)	2·1 (1·2)	2·5	3·7	4·1 (1·3)
DF625	0·5	0·3	0·23 (0·02)	2·5 (1·0)	2·7	4·5	5·7 (1·3)
DF723	1·0	2·0	1·53 (0·26)	1·0 (0·5)	1·1	2·7	3·0 (1·0)
DF686	1·0	0·3	0·33 (0·07)	4·6 (3·6)	5·0	6·9	7·8 (3·1)
DF668	0·5	0·3	0·17 (0·02)	1·4 (0·4)	1·9	2·4	4·6 (1·7)

When the interquaternary distance was altered, by giving n values from 1 to 6, the duration of paralysis was affected. With relatively long chains in which n was 5 (DF621) or 6 (DF635), a duration of action even more transient than that of suxamethonium was obtained as judged by tests in the cat, whilst with short chains as in the malonic (DF595) and succinic (DF593) acid esters the paralysis was intermediate in duration between that of suxamethonium and gallamine triethiodide. In rabbits there was virtually no gradation in recovery time with compounds where n varied between 1 and 4; other values of n were not examined. There was little difference in the times required for recovery from head drop after DF596 ($n=4$) and DF595 ($n=1$) although in the cat the duration of action of DF595 was considerably longer (Fig. 2). The rate of recovery

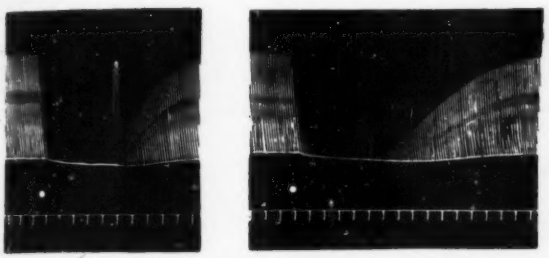


FIG. 2.—Cat. Maximal twitches of the gastrocnemius muscle elicited by indirect stimulation. Time, min. (*a*) 1 mg./kg. of DF596 was given intravenously. (*b*) 0.6 mg./kg. of DF595 intravenously.

following DF596 was comparable with that obtained with suxamethonium in the cat and dog, but this was not so in the rabbit, in which recovery after DF596 took place in about half the time needed after suxamethonium. As with suxamethonium, it was possible to produce a steady neuromuscular block by slow intravenous infusion of DF596 into cats and dogs, and recovery on stopping the infusion was quite rapid (Fig. 3).

In cats, a similar gradation of the effects of compounds upon blood pressure was also observed as the value of n was varied. Least hypotension was observed with the malonic acid ester DF595 ($n=1$), but as the number of methylene groups increased so also did the degree of hypotension produced. DF596 had a considerable hypotensive action in cats; weight for weight approximately 0.05 that of phenactropinium chloride (Robertson, Gillies and Spencer, 1957). In dogs, effects on blood pressure were much less marked. The oxalic acid ester (DF604) behaved anomalously, it was of very low activity in causing paralysis

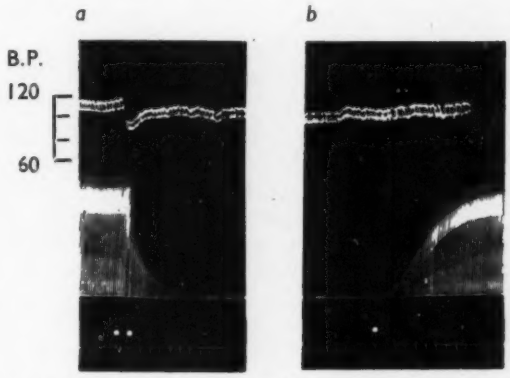


FIG. 3.—Dog anaesthetized with pentobarbitone. Upper tracing, carotid blood pressure in mm. Hg. Lower tracing, maximal twitches of the gastrocnemius muscle elicited by indirect stimulation. Time, min. (*a*) 0.1 mg./kg. of DF596 was injected at first dot. At second dot, an intravenous infusion of 0.015 mg./kg./min. of DF596 was started. (*b*) 45 min. later, the infusion of DF596 was stopped.

and gave a much greater fall in blood pressure than the malonic acid ester (DF595).

Derivatives of Other Esters

With compounds derived from acetyltropeïne (Series I) or benzoyltropeïne (Series II) in which m was 2, changes in duration of neuromuscular blocking action and effect on blood pressure were in the same direction as with the corresponding members of series derived from phenylacetyltropeïne (Series III) as n was increased from 2 to 6.

The most active benzoyltropeïne derivatives were the adipic [DF620, *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(3-benzoyloxytropanium bromide)] and pimelic [DF622, *NN'*-4,10-dioxo-3,11-dioxatridecamethylenebis(3-benzoyloxytropanium bromide)] acid esters. These were approximately equal in potency to the most active members of the phenylacetyltropeïne series. In general, however, benzoyltropeïnes were slightly longer acting than their phenylacetyl analogues. If Series II and III are compared, it can be seen that the most active benzoyltropeïnes have higher values of n than in the phenylacetyltropeïnes.

The three acetyltropeïne analogues prepared had low activity in the cat.

The adipic acid ester derived from β-phenylpropionyltropeïne [DF696, *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(3-β-phenylpropionyloxytropanium bromide)] closely resembled the corresponding ester derived from phenylacetyltropeïne in potency, duration and effect on blood pressure.

The Effect of Altering the Value of m

The effect of increasing the number of methylene groups in the chain between the ester groups and the quaternary nitrogen atoms from 2 to 3 was investigated only for the phenylacetyltropeïne derivatives (Series IV). This change tended to enhance the activity of the most potent members and at the same time to shorten the duration of action. For example, *NN'*-5,8-dioxo-4,9-dioxadodecamethylenebis(3-phenylacetoxytropanium iodide) (DF625) was considerably more potent and of shorter duration than *NN'*-4,7-dioxo-3,8-dioxadecamethylenebis(3-phenylacetoxytropanium bromide) (DF593). The potency of compounds did not depend only upon interquaternary distance. The positions of the ester groups were of importance in this respect. Compounds DF723 ($n=0$; $m=4$), DF625 ($n=2$; $m=3$) and DF596 ($n=4$; $m=2$), which all contained ten carbon atoms in the chain but differed in the positions of their ester groups, varied greatly in both potency and duration.

The Effects of Alterations at the Quaternary Nitrogen Atoms

When the relative positions of the methyl group and the linking chain on the quaternary tropane nitrogen were reversed (Series V), the properties of phenylacetyltropeïne derivatives were not much altered.

Miscellaneous Compounds

The possibility of substituting simpler groups for tropine esters in these compounds was investigated. Both *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(1-methylpiperidinium bromide) (DF616) and *NN'*-4,9-dioxo-3,10-dioxadodecamethylenebis(1-benzylpiperidinium bromide) (DF628), in which the tropeïne moiety was replaced by a piperidine ring, were less potent than DF596, and the mode of action of the compounds differed from that of other members of the series.

NN'-Decamethylenebis(3-phenylacetoxytropanium bromide) (DF41) was included because it enabled a comparison to be made between a bis-quaternary tropeïne compound with only methylene groups in the chain and another (DF593) of about the same interquaternary distance with ester groups also present in the chain.

The Mode of Action of Compounds

The neuromuscular block obtained with several of the more active compounds could be reversed by a subsequent injection of neostigmine or edrophonium, both in the anaesthetized cat and in the isolated mammalian nerve-muscle preparation. Compounds tested in this way are indicated in Table I.

All members of the series described here in which each quaternary nitrogen atom was present in a tropane nucleus produced a flaccid paralysis when injected into chicks. No contracture was observed when one of these compounds was added to the fluid bathing an isolated frog rectus abdominis muscle, but contractures due to acetylcholine were reduced or abolished.

Both DF616 and DF628 containing a piperidine ring in place of the tropeïne moiety showed some of the properties characteristic of depolarizing agents. DF616 gave a spastic paralysis in the chick apparently identical with that given by suxamethonium, tested at the same time. This differed from the paralysis due to DF628, which was spastic initially but subsequently gave way to flaccidity. On the frog rectus muscle, DF616 produced a contracture when added to the bath fluid whereas DF628 itself had no effect on the muscle but antagonized the action of acetylcholine.

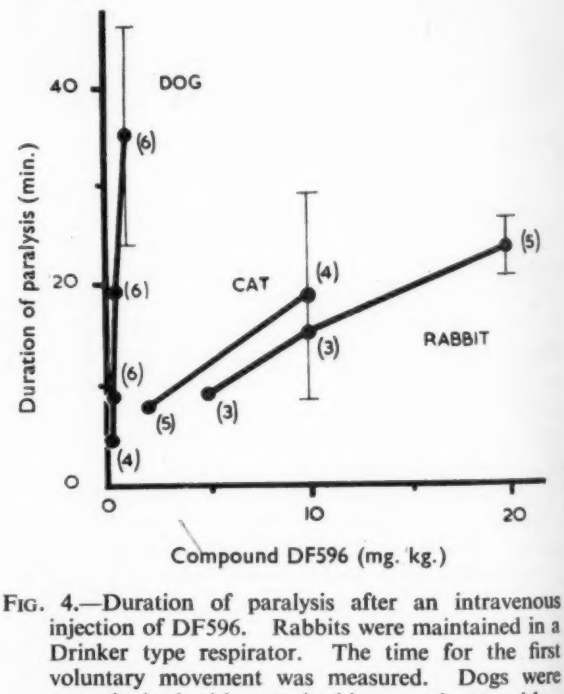


FIG. 4.—Duration of paralysis after an intravenous injection of DF596. Rabbits were maintained in a Drinker type respirator. The time for the first voluntary movement was measured. Dogs were anaesthetized with pentobarbitone and cats with a mixture of chloralose and urethane. Time for recovery of the indirectly-elicited gastrocnemius twitch to 50% of its pre-injection height was measured. Standard deviations are shown by vertical bars. Numbers of animals used are shown in brackets.

Duration of Action of Compounds in Different Species

Different species varied in their ability to inactivate DF596. Fig. 4 shows that rabbits can deal with about 15 mg./kg. and dogs about 0.5 mg./kg. in 20 min. Duration of action of DF596 was compared with that of tubocurarine and suxamethonium in mice by estimating the time elapsing after recovery of the righting reflex until animals were able to retain their hold on an almost vertical wire mesh screen. Recovery after DF596 was more rapid than after suxamethonium or tubocurarine (Table III). Confirmation of these results was obtained by determining the ratio of subcutaneous ED50 (paralysis) to the intravenous ED50 (paralysis) in mice. A high ratio would be expected if considerable metabolism of the compound had taken place before onset of paralysis. Table IV shows that the ratios for the relatively long-acting relaxants tubocurarine and gallamine triethiodide were 2.1 and 1.6 respectively whereas for the much shorter acting suxamethonium chloride the value was 9.7. The values of 32 and 29 obtained for DF595 and DF596 suggest that these compounds are even more rapidly inactivated than suxamethonium.

TABLE III

THE DURATION OF ACTION OF NEUROMUSCULAR BLOCKING AGENTS IN MICE

The values in col. 2 give the time after recovery of righting reflex until the animal was able to support itself on a near-vertical wire mesh screen after an intravenous infusion of the drug in sec. with s.d.

Compound (1)	(2)
Suxamethonium	108±18
Tubocurarine	285±190
DF596	16±18

TABLE IV

RATIO OF SUBCUTANEOUS ED50 TO INTRAVENOUS ED50 OF NEUROMUSCULAR BLOCKING AGENTS IN MICE

ED50, the quantity for paralysis in mice with 95% limits.

Compound	ED50 (mg./kg.)		Subcut. ED50 / I.V. ED50
	Subcutaneous	Intravenous	
Tubocurarine	0·094 (0·087–0·101)	0·045 (0·037–0·056)	2·1
Gallamine triethiodide	1·85 (1·56–2·20)	1·16 (1·02–1·31)	1·6
Suxamethonium	1·60 (1·46–1·74)	0·165 (0·132–0·207)	9·7
DF595	14·3 (13·4–15·3)	0·45 (0·38–0·53)	32
DF596	8·0 (7·1–9·0)	0·28 (0·26–0·31)	29

Effects of Compounds on the Isolated Human Foetal Phrenic Nerve-Diaphragm

This preparation was used to assess the likely potency of compounds in man. It was appreciated that the preparation might lead to erroneous conclusions for compounds destroyed *in vivo* only and that it was unlikely to be a suitable preparation for comparing compounds

TABLE V

RELATIVE POTENCY AND DURATION OF ACTION OF NEUROMUSCULAR BLOCKING AGENTS ON THE ISOLATED HUMAN FOETAL DIAPHRAGM

DF596 was used as the standard of comparison for potency. An asterisk indicates that the compound was antagonized by neostigmine. The relative duration of action is measured arbitrarily by the number of + : the greater the number the greater the duration of action.

Compound	No. of Estimates	Relative Potency	Relative Duration of Action	Approx. Conc. Giving 50% Reduction of Twitch in 5 Min. (μg./ml.)
Suxamethonium	3	>5	++	2–10
Tubocurarine	2		+++++	1–5
Gallamine triethiodide	1	1	++++	
DF595 ..	2	3*	++ to +++	10–20
DF596 ..	4	1*	+ to ++	20–80
DF668 ..	1	<1		

acting competitively with those exerting a depolarizing action. Different preparations were found to vary greatly in their recovery rates after the same compound. Our observations confirmed those of Buller and Young (1949), who found that, generally, recovery was slow so that potency was estimated by bracketing or matching responses. Values shown in Table V are all relative to DF596 compared on the same preparation. DF596 was considerably less potent than suxamethonium on this preparation, and duration of action was also somewhat less. DF595 was approximately 3 times as potent as DF596 and was of longer duration than suxamethonium.

The epimeric form of DF596 (DF668) did not appear to offer any advantages.

Discussion

The results clearly indicate that symmetrical bis-quaternary tropeïne compounds, in which two tropine esters were linked through their nitrogen atoms by a polymethylene chain containing two ester linkages, exhibited a neuromuscular blocking action of short duration which is reversible by neostigmine.

The phenylacetyltropeïne derivatives (Series III) in which n varies between 0 and 5 resembled the dicholine esters of $\alpha\omega$-dicarboxylic acids (I) prepared and tested by Bovet, Bovet-Nitti, Guarino, Longo and Fusco (1951) with regard to the influence which alterations in the value of

$$(CH_3)_3\overset{+}{N}\text{-}[CH_2]_2\text{-O-CO-}[CH_2]_n\text{-CO-O-}[CH_2]_2\text{-}\overset{+}{N}(CH_3)_3$$

I

n have on potency. The two series differed, however, in mode of action since the choline derivatives exerted a depolarizing action while the tropine compounds resembled tubocurarine in their behaviour. The side-effects of the members of the two series were also opposite in nature; increasing the value of n in the choline series increased hypertensive properties whereas similar changes in the tropine series increased hypotension.

Competitive action in neuromuscular blocking agents is usually associated with bulky terminal groups. All symmetrical bis-quaternary tropeïnes tested by us have behaved like competitive agents as judged by tests in chicks and on frog muscle. None has been reported by other workers to exert a depolarizing action. The swing from depolarizing towards a competitive action seen on replacing the *N*-methyl group in the piperidine analogue of DF596 (DF616) by a benzyl group (DF628) supports the idea that the bulk of the terminal cationic head is of importance.

From consideration of structure alone it was expected that the tropeïne derivatives would behave like tubocurarine. Comparison of results obtained by other workers for gallamine triethiodide, benzoquinonium, tubocurarine and laudexium suggests that with competitive neuromuscular blocking agents, the equivalent dose for man on a mg./kg. basis might be intermediate between those required for cat and mouse. With such agents the order of decreasing sensitivity is rabbit>cat>man>mouse. In laboratory animals, DF596 conforms to this pattern. The duration of action of DF596 relative to that of suxamethonium varied considerably in different species; it was approximately the same in cats, slightly shorter in dogs, much shorter in rabbits and mice and also somewhat shorter on the human foetal diaphragm. On this last preparation it was much less potent than suxamethonium, and, assuming that the preparation gives a valid estimate of activity in man, the potency of DF596 would be expected to be less than one fifth that of suxamethonium chloride; possibly only a tenth that of tubocurarine chloride.

It is clear that, with compounds of the type investigated here, marked species differences exist not only in potency, as is known to be so with most neuromuscular blocking agents, but also in duration of action. With DF596, the ratio of doses needed to paralyse in the most and least sensitive species examined was actually less than that found for tubocurarine in the same test, but duration of action in different species varied considerably.

The mechanism controlling the duration of action of members of this series was not investigated, but, by analogy with suxamethonium and other dicholine esters of $\alpha\omega$-dicarboxylic acids, it is reasonable to suppose that splitting of the chain by enzymatic hydrolysis may occur. The possibility of other mechanisms being involved, however, is shown by the failure of Hidalgo, Wilken and Seeberg (1959) to demonstrate hydrolysis of relatively short-acting hypotensive agents under conditions effective with acetylcholine. The relatively brief action of members of Series III in rabbits and the lack of gradation in this effect shown with different values of n leads to the suspicion that esterase activity at the chain is not the sole factor governing duration. DF41, which has only terminal ester links, was long acting in the cat but relatively short acting in rabbits. It is possible that the esterases capable of attacking terminal ester groups in addition to those effective in the chain play a part in the breakdown of these tropine esters; in rabbits this is quite likely

since the presence of an esterase in serum capable of splitting atropine and homatropine is well known (Ambache, 1955).

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INHIBITION OF MAST CELL DISRUPTION AND HISTAMINE RELEASE IN RAT ANAPHYLAXIS *IN VITRO*. COMPARISON WITH COMPOUND 48/80

BY

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In vitro anaphylactic reaction causes mast cell damage and histamine release from rat tissue. Histamine release is correlated with mast cell damage and both phenomena are simultaneously inhibited by various metabolic inhibitors, antipyretics, calcium lack and previous heating of the tissue at 45°. The mast cell damage produced by antigen in sensitized rat tissues is morphologically similar to that caused by compound 48/80, both agents causing extrusion of granules. Mast cell damage and histamine release induced by antigen or by compound 48/80 are inhibited alike by several substances and conditions. It is suggested that in rats the histamine-releasing mechanism of the antigen-antibody reaction in anaphylaxis is very similar to that of compound 48/80.

Liberation of histamine by antigen *in vitro* has been demonstrated in sensitized tissues of several species. Although histamine could be detected in relatively high amounts in plasma of shocked rats (Mota, 1957, 1958a) there are no data on histamine release by antigen from isolated sensitized tissues in this species. It is known that in guinea-pig anaphylaxis, histamine liberation is correlated quite well with mast cell damage, both these processes being inhibited in the same way by several agents (Mota, 1958b, 1959; Humphrey and Mota, 1959). Thus it seemed interesting to find out if isolated sensitized rat tissues would release histamine when in contact with antigen, whether this release was correlated with mast cell damage and how far the inhibitors of guinea-pig anaphylaxis (Mongar and Schild, 1957a, 1957b, 1958) would also affect rat anaphylaxis. Furthermore a comparative study of the effect of these same inhibitors on histamine release and mast cell disruption induced by compound 48/80 was also performed.

METHODS

Wistar rats of either sex, body weight 150 to 250 g., were used in all experiments. Crystalline bovine serum albumin and normal horse serum were used as antigens. Horse serum was dialysed against cold saline for 48 hr., filtered and stored under aseptic conditions. Four schedules of sensitization were used: (*a*) animals intraperitoneally injected with 1 ml. of alum-precipitated horse serum at 2-day intervals for three injections; (*b*) animals intraperitoneally injected with 1 ml. of horse serum plus 1 ml. of *Haemophilus pertussis* vaccine containing 20,000m. phase I organisms; (*c*) animals injected with 0.2 ml. of horse serum emulsified in Freund's adjuvant according to the technique of Lipton, Stone, and Freund (1956); (*d*) animals injected intraperitoneally with 1 ml. of a 1% solution of bovine serum albumin plus 1 ml. of *Haemophilus pertussis* vaccine containing 20,000m. phase I organisms.

The animals were used between the tenth and the twentieth day after sensitization. They were bled under ether anaesthesia and their skin and mesentery removed.

Removal and Treatment of Skin.—The abdominal skin was shaved with an electrical clipper, cut in pieces of about 1 sq. cm., carefully removed from the abdominal wall and kept in cold Tyrode solution. Each piece of skin was used as a sample. The samples were then shaken in 6 ml. stoppered flasks at 37° for 15 min. with 3.6 ml. Tyrode solution or Tyrode containing the required concentration of inhibitor. After this treatment either bovine serum albumin, horse serum, or compound 48/80 was added. Bovine serum albumin was added to give a final concentration of 1 mg./ml., horse serum to give a 10% solution, and compound 48/80 to give 0.1 mg./ml.

The tissue was then incubated for a further period of 15 min. after which the skin was transferred to N hydrochloric acid, cut into very small pieces, boiled for 2 or 3 min., and kept at 4° until assayed. The incubation solution was acidified with one drop of concentrated hydrochloric acid, boiled and kept

at 4°. As a control to each experiment samples of sensitized skin were incubated in Tyrode solution and samples of non-sensitized skin were incubated with the same concentration of each antigen.

Removal and Treatment of Mesentery.—The mesentery was dissected away from the small intestine and cut into several small pieces which were dropped in cold Tyrode solution. One or two of these pieces were used as a sample. The samples were incubated in the conditions already described for the skin samples and then transferred to the fixative or to N hydrochloric acid. The incubation solution was acidified with one drop of concentrated hydrochloric acid, boiled and kept at 4°.

Histamine Assay.—The extracts and incubation solutions were neutralized with sodium hydroxide, and when necessary diluted with Tyrode solution to the required volume. The histamine content was estimated on the atropinized guinea-pig ileum using histamine acid phosphate as a standard. In order to exclude any non-histamine component in the solution they were retested after inhibition of histamine by mepyramine 10^{-8}. Histamine release in controls was subtracted from that released by antigen or compound 48/80. Histamine release was expressed as a percentage of the total tissue histamine.

Mast Cell Observation.—The skin was fixed by immersion in 50% aqueous ethanol containing 10% formaldehyde and 5% acetic acid. Fixation was allowed to proceed overnight and frozen sections 50 μ thick were stained by 1% toluidine blue. The mesentery was fixed and stained in 10% formaldehyde containing 1% toluidine blue and 0.1% acetic acid, and examined as a whole-amount preparation. The number of disrupted mast cells in the mesentery was counted and calculated as a percentage of the total (500 cells being counted).

Inhibitor Solutions.—All inhibitors were dissolved in Tyrode solution and when necessary sodium hydroxide was used to help solution. The *p*H was then determined electrometrically and adjusted with hydrochloric acid to 7.8 in all solutions.

Results

Histamine Release from Rat Skin by Antigen.—Samples of sensitized skin were incubated with antigen and the percentage of histamine released was determined. The results are shown in Tables I and II. It can be seen that antigen released histamine from sensitized rat skin and that this release varied from rat to rat but was very constant in samples of abdominal skin taken from the same animal. Results of control pieces show that the spontaneous histamine release was small and that none of the antigens released histamine from non-sensitized rat skin.

Histamine Release from Rat Mesentery by Antigen.—Histamine release from mesentery is

TABLE I

HISTAMINE RELEASE BY ANTIGEN FROM SIX SAMPLES OF SKIN FROM A SENSITIZED RAT

Control Release μg.	Antigen Release μg.	Residue μg.	% Total Tissue Histamine Released	
			Control	Antigen
0·03	2·0	11	0·2	15
0·16	2·7	14	0·9	16
0·20	1·8	12	1·4	13
0·20	1·9	13	1·3	12
0·05	2·2	13	0·3	14
0·15	2·1	14	0·9	13

TABLE II

HISTAMINE RELEASE BY ANTIGEN FROM SENSITIZED RAT SKIN *IN VITRO*

The controls represent the percentage release from a sensitized piece of rat skin incubated with Tyrode solution (A) and from a non-sensitized piece of rat skin incubated with the antigen (B).

Rat	Group	Days After Sensitization	% Total Tissue Histamine Released		
			Antigen	Controls	
				A	B
1	A	20	18	1	0·8
2	A	20	9	1	0·7
3	A	18	6	1	2
4	A	16	9	3	1
5	A	16	11	1	1
6	A	19	10	1	1
7	A	19	7	1	2
8	B	12	13	2	2
9	B	12	25	1	0·9
10	B	12	14	2	3
11	B	15	16	1	1
12	B	15	11	1	2
13	B	12	23	2	1·8
14	B	12	20	2	2
15	B	11	16	1	0·9
16	B	13	8	0·7	0·6
17	C	13	10	0·5	1
18	C	10	14	2	2·1
19	C	15	18	1	1
20	C	15	15	0·9	0·8
21	D	13	20	3	2
22	D	13	11	4	3
23	D	13	23	1	2
24	D	13	18	2	1
25	D	14	24	3	3

TABLE III
HISTAMINE RELEASE AND MAST CELL DISRUPTION BY ANTIGEN IN RAT MESENTERY *IN VITRO*
Each figure refers to one animal.

Method of Sensitization. (See Methods)	Histamine Release %		Mast Cell Disruption %	
		Mean		Mean
a	22, 18, 28, 30	25	10, 11, 12, 15, 16	13
b	37, 40, 35, 36, 29, 31	35	10, 68, 51, 28, 48, 21	36
c	18, 28, 17	31	23, 10, 8, 46	22
d	17, 28, 29, 26, 21	19	32, 21, 29, 44, 61	37

shown in Table III. It can be seen that the percentage of histamine released was greater than that released from the skin possibly due to the thinness of this tissue, allowing an almost immediate contact of antigen with the cells. The amount of the spontaneously released histamine from mesentery was usually too small to be detected.

Mast Cell Disruption.—Microscopic examination of skin and mesentery after *in vitro* contact with antigen showed constant and definite alterations of mast cells, very similar to those previously described in the intact animal (Mota, 1953, 1957); both conditions were characterized by extrusion of granule similar to that caused by compound 48/80 (Mota, Beraldo, and Junqueira, 1953). It must be emphasized that this kind of alteration was present only in sensitized tissues after contact with antigen, and was absent from non-sensitized tissues incubated with antigen; it was easily distinguishable from artificial disruption. The percentage of mesenteric mast cells showing extrusion of granules after contact with antigen is shown in Table III. It can be seen that a variable number of mast cells were disrupted and that some of them preserved their usual morphology.

Effect of Metabolic Inhibitors on Histamine Release by Antigen or Compound 48/80.—Samples of sensitized skin were used in these experiments. The effects of sodium iodoacetate, sodium *p*-chloromercuribenzoate, sodium *o*-iodosobenzoate, *N*-ethylmaleimide and sodium cyanide were investigated. The results of these experiments are shown in Table IV and summarized in Fig. 1. It can be seen that all metabolic inhibitors

TABLE IV
EFFECT OF VARIOUS METABOLIC INHIBITORS ON HISTAMINE RELEASE BY ANTIGEN AND COMPOUND 48/80 FROM RAT SKIN

Releaser	Inhibitor	Molar Concentration	% Total Tissue Histamine Released by		% Inhibition
			Releaser Alone	Releaser + Inhibitor	
Antigen	Sodium iodoacetate	0·001	11	1	
			16	5	
			12	1	80
			15	4	
			23	2	
Compound 48/80	,, ,,	0·001	19	2	
			35	4	
			14	1	90
			16	2	
			11	1	
Antigen	Sodium *p*-chloromercuribenzoate	0·001	11	3	
			16	2	
			12	3	87
			15	1	
			23	2	
Compound 48/80	,, ,,	0·001	28	2	
			19	2	92
			14	1	
			35	3	
Antigen	*N*-Ethylmaleimide	0·001	11	1	
			16	1	
			12	2	93
			15	1	
			23	1	
			9	—	
Compound 48/80	,, ,,	0·001	25	3	
			21	4	
			27	1	96
			35	1	
			19	2	
Antigen	Sodium *o*-iodosobenzoate	0·005	11	1	
			16	3	
			12	2	
			15	1	90
			23	1	
			9	1	
			20	1	
Compound 48/80	,, ,,	0·005	28	2	
			19	2	
			14	1	96
			35	1	
			19	1	
Antigen	Sodium cyanide	0·005	11	2	
			16	2	93
			12	1	
Compound 48/80	,, ,,	0·005	21	5	
			25	3	86
			22	1	

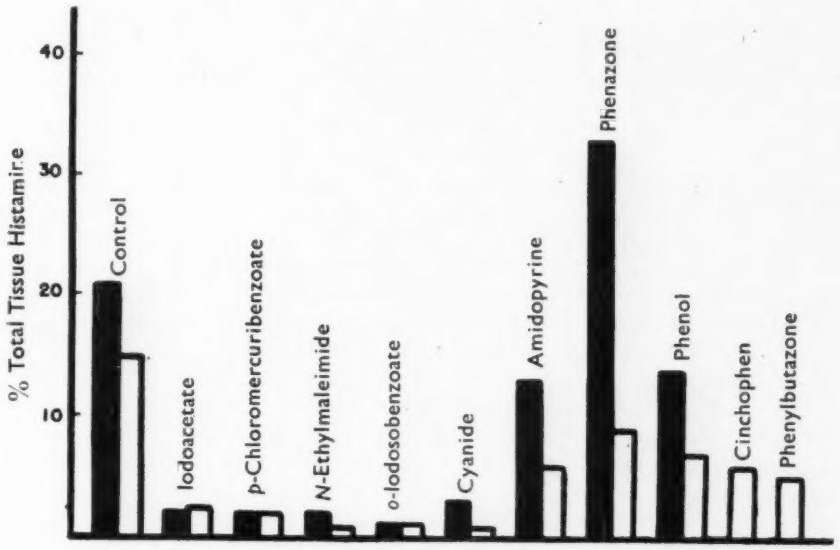

FIG. 1.—Effect of metabolic inhibitors and antipyretics on histamine release by antigen (white columns) or compound 48/80 (black columns) from rat skin.

used abolished almost completely the histamine release either by antigen or compound 48/80.

Effect of Metabolic Inhibitors on Mast Cell Damage by Antigen or Compound 48/80.—When pieces of sensitized mesentery were previously incubated with the various metabolic inhibitors, addition of antigen or compound 48/80 caused practically no mast cell damage as can be seen in Table V.

TABLE V

EFFECT OF METABOLIC INHIBITORS ON MAST CELL DISRUPTION *IN VITRO* CAUSED BY ANTIGEN OR COMPOUND 48/80, IN RAT MESENTERY

The values represent % mast cell disruption caused by antigen or compound 48/80 alone, and in the presence of inhibitor.

Inhibitor/ Releaser	Sodium Iodo-acetate	Sodium *o*-Iodoso-ben-zoate	Sodium *p*-Chloro-mercuri-benzoate	*N*-Ethyl-male-imide	Sodium Cyanide
Antigen 21	0	0	0	0	0
,, 38	0	1	2	0	11
,, 67	0	0	5	0	12
,, 18	0	0	0	0	0
,, 10	0	0	0	0	0
,, 68	0	0	2	0	3
Compound 48/80 62	0	2	2	0	5
,, 73	6	1	1	0	3
,, 56	0	0	0	0	2
,, 45	0	2	0	0	0
,, 28	0	0	0	0	0

Effect of Antipyretics on Histamine Release by Antigen or Compound 48/80.—Samples of sensitized skin were used in these experiments. The effects of amidopyrine, phenazone, cinchophen, and phenylbutazone were investigated. It was observed that the addition of compound 48/80 to a solution of cinchophen or phenylbutazone caused a precipitate and these antipyretics were not tried with 48/80. The results of these experiments are shown in Table VI and summarized in Fig. 1. The antipyretics caused an inhibition of histamine release by both antigen and compound 48/80 with the exception of phenazone, which although reducing histamine release by antigen increased histamine release by compound 48/80.

Effect of Calcium Lack on Histamine Release and Mast Cell Damage by Antigen or Compound 48/80.—Pieces of sensitized mesentery were incubated in calcium-free Tyrode solution containing 0.01% sodium dihydrogen edetate (EDTA) for 15 min. and then antigen (bovine serum albumin) or compound 48/80 was added. As can be seen in Fig. 2, both histamine release and mast cell damage were prevented by calcium lack.

Effect of Previous Heating of the Tissue to 45°.—Pieces of sensitized mesentery were kept at 45° for 5 min. and restored to 37°. Subsequent incubation of the mesentery with antigen (bovine serum albumin) or compound 48/80 showed that this treatment inhibited both histamine release and

TABLE VI
EFFECT OF VARIOUS ANTIPYRETICS ON HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80 FROM RAT SKIN

Releaser	Inhibitor	Molar Concentration	% Total Tissue Histamine Released by		% Inhibition
			Releaser Alone	Releaser + Inhibitor	
Antigen	Amidopyrine	0·01	18	10	
			5	2	
			9	4	45
			9	5	
			10	6	
			16	9	
Compound 48/80	,,	0·01	25	18	
			21	10	38
			27	15	
			11	11	
Antigen	Phenazone	0·01	25	25	
			15	5	
			9	6	29
			9	7	
			10	8	
			16	8	
Compound 48/80	,,	0·01	25	21	
			21	45	0
			27	34	(Histamine
			11	22	release
			24	35	increased
			17	45	from 21% to 34%)
Antigen	Phenol	0·01	11	5	
			16	9	
			12	4	50
			15	6	
			11	5	
			23	12	
Compound 48/80	,,	0·01	25	14	
			21	23	
			27	13	36
			11	5	
			24	15	
Antigen	Cinchophen	0·01	18	10	
			5	3	
			9	9	45
			9	3	
			10	4	
			16	7	
,,	Phenylbutazone	0·005	18	8	
			5	3	
			9	4	45
			9	6	
			10	6	
			16	8	

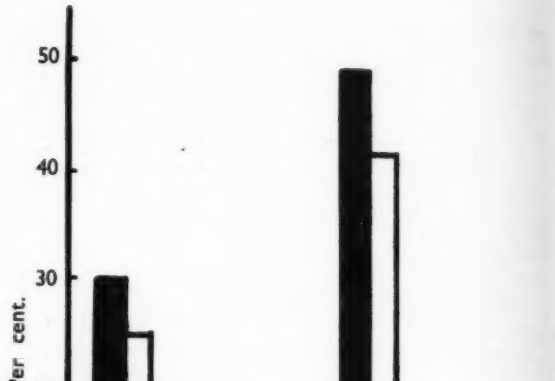

FIG. 2.—Effect of Tyrode solution (A), calcium lack (B) or previous heating of the tissue at 45° (C) on histamine release (black columns) and mast cell disruption (white columns) induced by antigen or compound 48/80 in sensitized mesentery.

mast cell damage by antigen or compound 48/80. The results of these experiments are shown in Fig. 2.

DISCUSSION

It can be seen from these results that antigen releases histamine from sensitized isolated rat tissue as it does in other species and that histamine liberation is well correlated with mast cell damage in rat anaphylaxis.

The present paper also shows that the several substances and conditions known to inhibit histamine release and mast cell damage in guinea-pig anaphylaxis are equally effective in the rat. Furthermore it is interesting to observe that in rats the mast cell damage induced by antigen or compound 48/80 is morphologically similar and that the histamine-releasing effect of both is inhibited by various agents. Thus there seems to be a close parallelism between the histamine-releasing mechanism of antigen and compound 48/80 in the rat. This fact suggests that in this species compound 48/80 may provide a good model for studying histamine release in anaphylaxis although the results of the experi-

ments with phenazone seem to suggest that the two reactions are not identical.

It is known that in the guinea-pig previous heating of the tissue at 45° causes a persistent inactivation of the histamine-releasing mechanism by antigen but does not interfere with the histamine-releasing mechanism of chemical histamine liberators (Mongar and Schild, 1957b). However, the present results show that in the rat this effect is not specific for anaphylaxis and that the tissue component inactivated at 45° is also required in the reaction leading to histamine liberation by compound 48/80. Furthermore the experiments with calcium lack suggest that in this species calcium ions are necessary for histamine release either by antigen or by compound 48/80.

The finding that various sulphydryl blocking agents inhibit mast cell damage by compound 48/80 had already been observed by Junqueira and Beiguelman (1955), and the present observations confirm their results. The inhibitory effects of *p*-chloromercuribenzoate, *o*-iodosobenzoate, iodoacetate, and *N*-ethylmaleimide suggest that free sulphydryl groups are implicated in the chain reaction leading to histamine release by antigen-antibody reaction as well as by compound 48/80 in the rat.

It is known that there is some analogy between the effects of the histamine releasers and the effects of antigen-antibody reaction in anaphylaxis. Furthermore it was shown that the histamine released by the antigen-antibody reaction and that which can be released by chemical histamine liberators have at least one common site of origin, both agents causing mast cell damage (Mota, 1958b, 1959). On the other hand there are also differences between the histamine releasing mechanism of these two agents. Thus while histamine liberators can release histamine from mast cell granules, the antigen-antibody reaction releases histamine only from intact cells (Copenhaver, Nagler, and Goth, 1953; Mongar and Schild, 1956; MacIntosh, 1956). Furthermore, several agents which will inhibit mast cell damage and histamine release by antigen will not inhibit mast cell damage and histamine release by chemical histamine liberators in guinea-pigs (Mongar and Schild, 1957a, 1957b, 1958; Mota, 1958b, 1959). These results led to the conclusion that the histamine release mechanism of the antigen-antibody reaction and that of chemical releasers was quite different. However, this conclusion does not seem to apply to all species since at least in the rat there is a very close similarity between the histamine release mechanism of the antigen-antibody reaction and that of compound 48/80. This does not mean that the histamine release mechanism of these two agents is necessarily identical. For instance, if all inhibitors acted on one step of the histamine release mechanism common to antigen-antibody reaction and compound 48/80 both these agents could be inhibited in the same way, and yet other stages in their mechanism of action could be quite different. It is possible that this common step is the activation of a cellular enzyme system as suggested by Rocha e Silva (1959). In regard to this possibility it is interesting to consider Benditt's findings (1956) showing that rat mast cells are rich in a chymotrypsin-like enzyme, and the more recent observations of Braun-Falco and Salfed (1959) showing that rat mast cells are rich in leucine-aminopeptidase and that rupture of these cells is simultaneous with release of this enzyme.

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THE MODE OF NEUROMUSCULAR BLOCKING ACTION OF CHLORPROMAZINE

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The inhibitory action of chlorpromazine on skeletal muscle has been studied with isolated preparations. In the nerve-muscle preparations of the frog sartorius and the rat diaphragm, the twitch responses to indirect stimulation are much more strongly depressed by chlorpromazine than those to direct stimulation. The conductivity of the nerve trunk is unaffected. The contractures of the frog rectus abdominis muscle caused by acetylcholine are depressed by chlorpromazine,but the contractures due to KCl are not influenced. Larger doses of chlorpromazine cause contracture by themselves, and this cannot be prevented by tubocurarine. In the sartorius muscle of the toad, the depolarization due to acetylcholine is reduced by chlorpromazine. The paralysing action of chlorpromazine adds to that of tubocurarine, and is antagonized to some extent by eserine or neostigmine. Muscles treated with chlorpromazine do not completely recover on washing. High concentrations of chlorpromazine depress the release of acetylcholine by motor-nerve stimulation, although they do not affect the enzymic synthesis of acetylcholine by acetone-dried powder of guinea-pig brain. The differences between the neuromuscular block produced by chlorpromazine and that by tubocurarine are discussed.

Several papers have been published concerning the action of chlorpromazine on striated muscle. Huidobro (1954) observed that chlorpromazine did not modify the twitch of cat quadriceps muscle stimulated through its nerve. Dobkin, Gilbert, and Lamoureux (1954) reported that chlorpromazine did not block the neuromuscular junction in either frog or cat sciatic-gastrocnemius preparation.

Contrarily, many authors state that chlorpromazine inhibits striated muscle. Kopera and Armitage (1954) observed that chlorpromazine depressed the response of the gastrocnemius muscle of cats when stimulated either directly or indirectly through the sciatic nerve. They, as well as Burn (1954), considered that the action of chlorpromazine was not curare-like but was a direct action on the muscle. On the other hand, Ryall (1956) demonstrated that chlorpromazine could inhibit neuromuscular transmission in the isolated phrenic nerve-diaphragm preparation of rabbits, and Wislicki (1958) obtained the same result with the sciatic-gastrocnemius preparation of cats. Ryall (1956) suspected that chlorpromazine paralysed the muscle by decreasing acetylcholine synthesis at the motor nerve endings, but this idea has been rejected recently by Otubo (1959). The latter author stated that chlorpromazine at first stimulated the endplate membranes and later inhibited them.

In view of these conflicting results, we decided to perform experiments to throw some light upon the mode of action of chlorpromazine on striated muscle.

METHODS

Isolated preparations of striated muscle were used throughout so as to avoid interfering factors such as the actions of chlorpromazine on the central nervous or the cardiovascular systems.

Sartorious Muscle of the Frog.—The sartorius nerve muscle preparation of *Rana tigerina* was dissected out and set up in an organ bath containing 50 ml. of frog Ringer solution kept at room temperature and constantly oxygenated. Indirect and direct single electric shocks (rectangular in shape, supramaximal and of 0.5 and 5 msec. durations respectively) were applied once every 10 sec. The stimulator was a Model 751 by Arthur H. Thomas Co. The isotonic twitches of the muscle were recorded on smoked paper.

Diaphragm of the Rat.—The phrenic nerve-diaphragm preparation of the rat (Bülbring, 1946) was suspended in 50 ml. Tyrode solution, kept at 37° to 38° and constantly oxygenated with 95% O_2 and 5%

CO_2. Contractions were caused and recorded in the manner described for the sartorius muscle of the frog.

Rectus Abdominis Muscle of the Frog.—The rectus abdominis muscle of *R. tigerina* was excised and mounted in a 5 ml. bath containing frog Ringer solution at room temperature. Dose-response curves were obtained by applying acetylcholine in concentrations of 10^{-6}, 10^{-5}, 10^{-4}, and 10^{-3} g./ml. and recording the contractures on smoked paper. The contracture produced by 10^{-3} g./ml. acetylcholine was considered as the maximum response. The dose-response curves to KCl were produced similarly.

Depolarization of the Toad Sartorius Muscle.—A method similar to that of Fatt (1950) was used. The sartorius muscle of the toad (*Bufo melanostictus*) was mounted in a glass chamber containing 50 ml. of frog Ringer solution. An electrode was placed at the pelvic end and another near the bottom of the chamber in contact with the solution. In order to determine the position of maximum density of endplates, a preliminary record was obtained of the potential differences along the surface of each muscle preparation in the following manner: the level of the Ringer solution in the bath was lowered stepwise by draining 1 ml. away each time, and the potentials were measured by means of a potentiometer. With part of the muscle immersed in solution, the potential difference recorded was that between the upper end and a point on the muscle at the surface of the bathing solution. This procedure was repeated after substituting a solution of acetylcholine (8×10^{-6} g./ml.) for the Ringer. The point on the muscle surface which gave the maximum potential difference before and after using acetylcholine represented the focus of maximum endplate density. The depolarization by acetylcholine at the region of maximum endplate density was measured again, and the value was taken as 100%. The same procedure was repeated at 15 min. intervals after the application of chlorpromazine.

Estimation of Acetylcholine Release from the Rat Diaphragm.—A sector of the diaphragm, with phrenic nerve attached but without ribs, was suspended in a 3 ml. bath in Krebs bicarbonate solution containing 5×10^{-6} g./ml. neostigmine at 37°. After tetanizing at 25 shocks/sec. for 20 min. the fluid was withdrawn and assayed for acetylcholine by its depressor effect on the blood pressure of the anaesthetized rat (Straughan, 1958). Since the resting output of acetylcholine was negligible, no correction for this was made.

Acetylcholine Synthesis by Guinea-pig Brain.—The acetone-dried powder from guinea-pig brain was prepared and incubated at 37° with the reaction mixture of Feldberg and Hebb (1947). The acetylcholine produced was assayed on the frog's rectus abdominis muscle according to the method of Feldberg and Mann (1945).

The drugs used were chlorpromazine hydrochloride (Wintermin, Shionogi), tubocurarine chloride (Intocostrin-T, Squibb), acetylcholine chloride (Ovisot, Daiichi), neostigmine methylsulphate (Vagostigmine, Shionogi), and eserine salicylate (Merck). Concentrations of drugs are expressed as weights of salts, or as μM. or mM.

RESULTS

Sartorius Muscle of the Frog

The muscle contractions elicited by indirect stimulation were depressed markedly by chlorpromazine in concentrations of 30 to 50 μM. (11 to 18 μg./ml. of chlorpromazine hydrochloride) after an initial slight augmentation. The responses to direct stimulation also suffered inhibition which was, however, much smaller (Fig. 1). When a segment of the nerve trunk between the muscle and the electrodes was soaked in 10 to 100 μM. solutions of chlorpromazine, no change in the amplitude of contraction occurred for more than 1 hr. Similar results were obtained in a few experiments with sciatic nerve-gastrocnemius preparations of the frog.

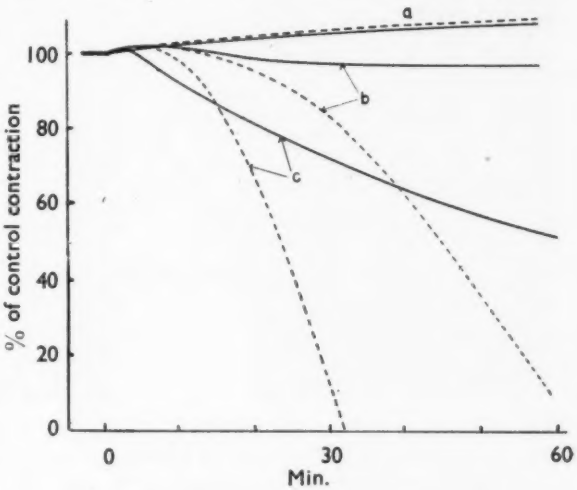

FIG. 1.—Frog, motor nerve-sartorius muscle. Amplitudes of single twitches in response to supramaximal stimulation. Solid line, direct stimulation; broken line, indirect stimulation. **a**, 10 μM.; **b**, 30 μM.; and **c**, 50 μM. of chlorpromazine given at zero time.

These results indicate that chlorpromazine exerts primarily a neuromuscular blocking action, plus a smaller action on the muscle fibres.

The slight augmentation of the contractions by 10 μM. of chlorpromazine and the initial transient augmentation after 30 to 50 μM. might have been due to the mild anticholinesterase activity of this drug (Erdös, Baart, Shanor, and Foldes, 1958).

The diminished contractions of this preparation could usually be restored by repeated washing with

Ringer's solution, even after a complete block had occurred. When the blocking action was fully developed the tetanic contraction caused by repetitive stimulation was no longer sustained, and no post-tetanic potentiation was found (Fig. 2).

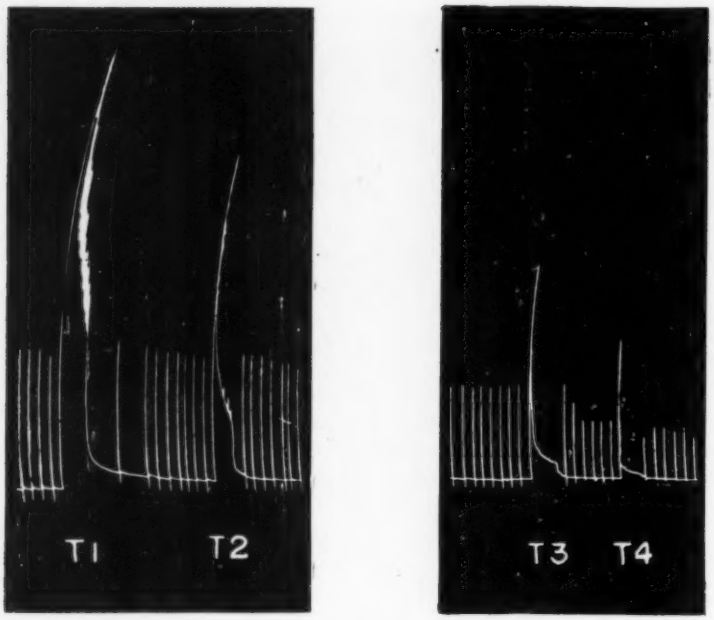

FIG. 2.—Frog, motor nerve-sartorius muscle. Tetanic stimulations 15 min. (left) and 20 min. (right) after application of 50 μM. chlorpromazine. Pulse frequency: 60/sec. (**T1** and **T3**) and 120/sec. (**T2** and **T4**).

Neostigmine and eserine (2×10^{-7} to 5×10^{-6} g./ml.) exerted a slight antagonistic effect. Adrenaline (5×10^{-7} to 5×10^{-6} g./ml.) was totally ineffective. The paralytic potencies of a dose of chlorpromazine, of an equivalent dose of tubocurarine and of the mixture of half-doses of the two agents were approximately equal, judging from the time required for complete paralysis.

Phrenic Nerve-Diaphragm Preparation of the Rat

Chlorpromazine in concentrations of 10 to 30 μM. reduced the muscle responses to indirect stimulation, after a brief augmentation. With a 40 μM. solution, a complete neuromuscular block was observed within 30 min., while the muscle still responded well to direct stimulation. Repetitive stimulation usually caused a well-sustained tetanic contraction (Fig. 3).

Washing sometimes retarded the blocking process, but the amplitude of twitches never recovered appreciably. Larger doses of chlorpromazine tended to provoke contracture of the diaphragm. Our results are in agreement with those of Ryall (1956). Eserine and neostigmine were only feebly effective unless applied early, when they generally enhanced the twitches (Fig. 4).

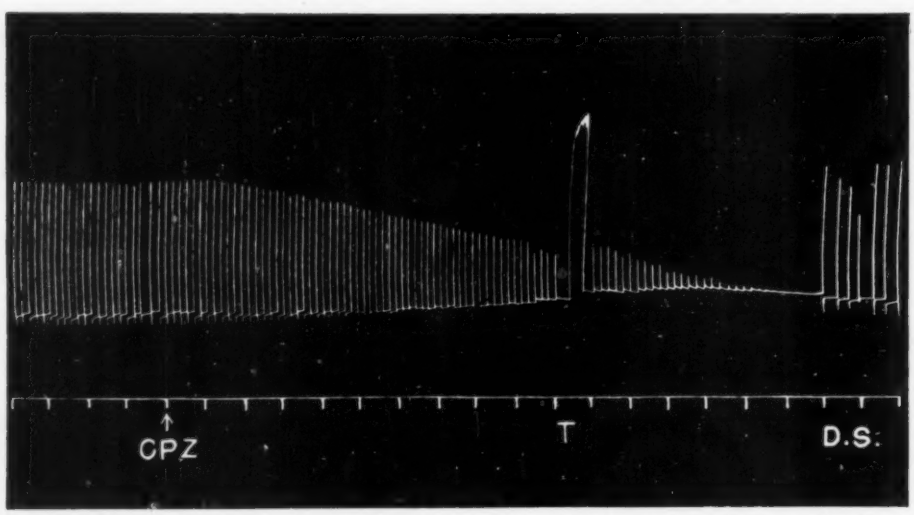

FIG. 3.—Rat, phrenic nerve-diaphragm. Supramaximal indirect stimulation. **CPZ**, 40 μM. chlorpromazine, tetanic stimulation at **T**. Direct stimulation at **D.S.** Time in min.

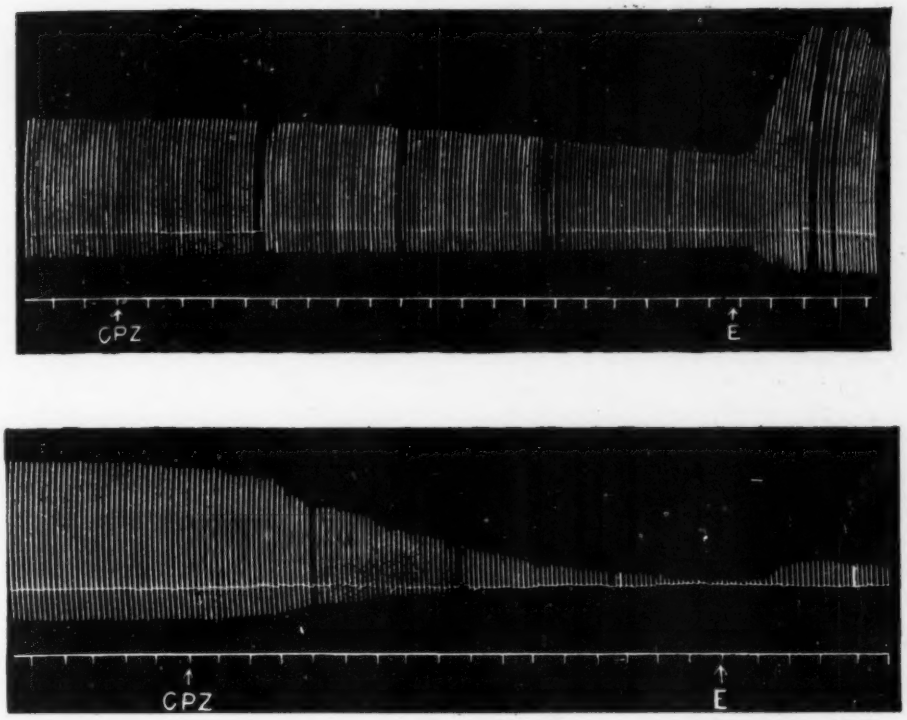

FIG. 4.—Rat, phrenic nerve-diaphragm. **CPZ**, chlorpromazine 30 μM. (above) and 50 μM. (below). Eserine 10^{-6} g./ml. at **E**. Time in min.

Rectus Abdominis Muscle of the Frog

The amplitudes of contracture caused by acetylcholine decreased after treatment of the muscle with solutions of chlorpromazine in concentrations higher than 0.1 μM. This effect was slower and weaker, on the molar basis, than that of tubocurarine. The action proceeded gradually and approached completion in 60 min.

The higher the concentration of acetylcholine, the more the response to it was depressed, and repeated washing did not lessen the depression appreciably (Fig. 5). Thus the action of chlorpromazine is at least partly irreversible.

On the other hand, chlorpromazine showed an approximately additive effect when used in combination with tubocurarine (Fig. 6). The addition of eserine or neostigmine completely antagonized chlorpromazine as far as the responses to low concentrations of acetylcholine were concerned, whereas the responses to higher concentrations of acetylcholine were depressed (Fig. 5). This suggests a mode of action different from that of tubocurarine.

Responses to KCl were not depressed by chlorpromazine (Fig. 7), since Ringer's solution alone after 1 hr. flattened the dose-response curve as much as chlorpromazine did. This was confirmed by the observation that, at the end of the tests with acetylcholine, the addition of an excess of KCl (300 mM.) still produced a maximum contracture.

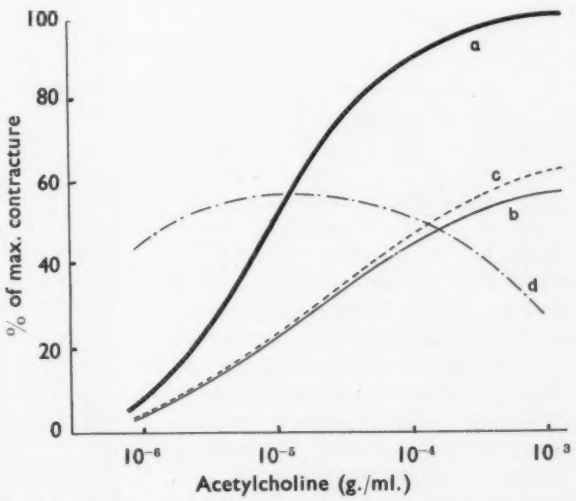

FIG. 5.—Frog, rectus abdominis. Responses to acetylcholine. **a**, Control. **b**, 2 μM. chlorpromazine for 60 min.. **c**, 2 μM. chlorpromazine for 60 min. followed by washing for 60 min. **d**, 2 μM. chlorpromazine and 10^{-6} g./ml. neostigmine for 60 min.

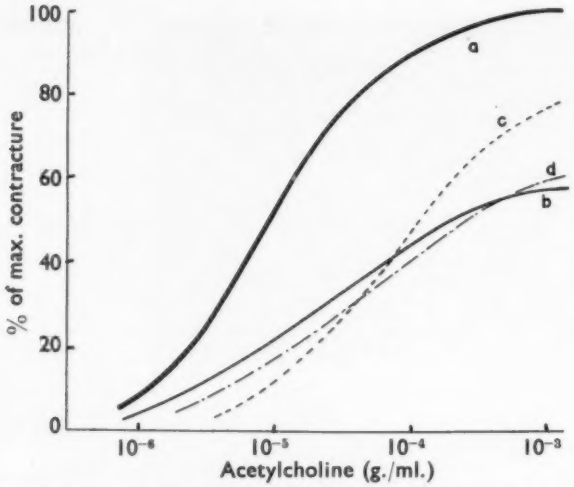

FIG. 6.—Frog, rectus abdominis. Responses to acetylcholine. **a**, Control. **b**, 2 μM. chlorpromazine for 60 min. **c**, 0.5 μM. tubocurarine for 60 min. **d**, 1 μM. chlorpromazine and 0.25 μM. tubocurarine for 60 min.

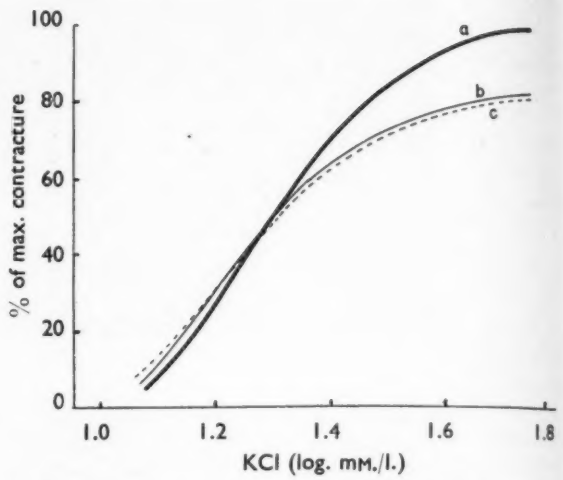

FIG. 7.—Frog, rectus abdominis. Response to KCl. **a**, Control. **b**, 2 μM. chlorpromazine for 60 min. **c**, Frog-Ringer for 60 min.

High concentrations of chlorpromazine caused contracture of the rectus muscle, which relaxed only very slowly on washing (Fig. 8). Previous treatment of the muscle with tubocurarine (4×10^{-5} g./ml. for 30 min.) had no influence on it, as Ryall (1956) observed for the diaphragm of the rabbit. The contracture produced by chlorpromazine, therefore, does not demonstrate an endplate depolarizing action of this agent, but seems to require some other explanation.

Depolarization in the Toad Sartorius Muscle by Acetylcholine

Chlorpromazine in 5 to 50 μM. solutions did not itself cause depolarization, but greatly diminished the magnitude of the depolarization caused by acetylcholine. The effect could be eliminated very slowly by washing several times unless large doses were used. Neostigmine reversed the inhibition effectively (Fig. 9). This experiment provided further evidence that chlorpromazine exerted an important inhibitory action on the motor endplates.

Acetylcholine Release by Motor-nerve Stimulation

As shown in Table I, the release of acetylcholine by motor-nerve stimulation was greatly reduced by 50 μM. of chlorpromazine. In one experiment (Expt. No. 3), it was completely abolished 1 hr. after the application of chlorpromazine. When 10 μM. of chlorpromazine was applied, however, no constant results were obtained ; in one experiment

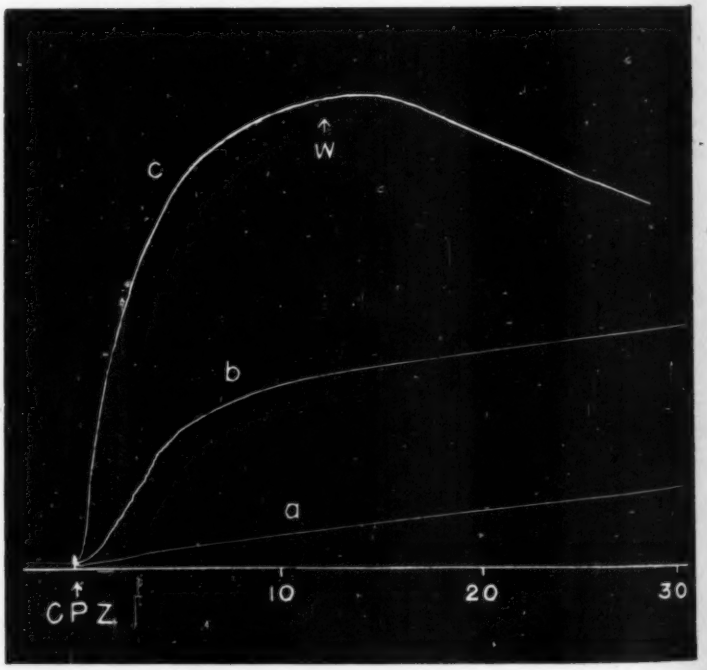

FIG. 8.—Frog, rectus abdominis. Contracture caused by chlorpromazine (CPZ), **a**, 10 μM., **b**, 100 μM., **c**, 1 mM. Washing at W. Time in min.

TABLE I

ACETYLCHOLINE OUTPUT OF THE RAT EXCISED DIAPHRAGM PREPARATION BEFORE AND AFTER THE ACTION OF CHLORPROMAZINE

There was 20 min. between the first and second periods of each control experiment.

Expt. No.	Acetylcholine Output (ng./20 min.)				Concentration of Chlorpromazine (μM.)
	Before Chlorpromazine		After Chlorpromazine		
	1st Period	2nd Period	30 Min.	60 Min.	
1	39	36	27	10	50
2	41	45	23	10	50
3	37	41	13	0	50
4	49	45	45	49	10
5	27	30	23	18	10

no reduction of acetylcholine released was found whereas in another experiment the amount of acetylcholine released was reduced by about 40% 60 min. after the application of chlorpromazine.

Synthesis of Acetylcholine by the Guinea-pig Brain

The results of a typical test are shown in Table II. It will be seen that chlorpromazine, even in concentrations as high as 100 μM., did not affect appreciably the synthesis of acetylcholine.

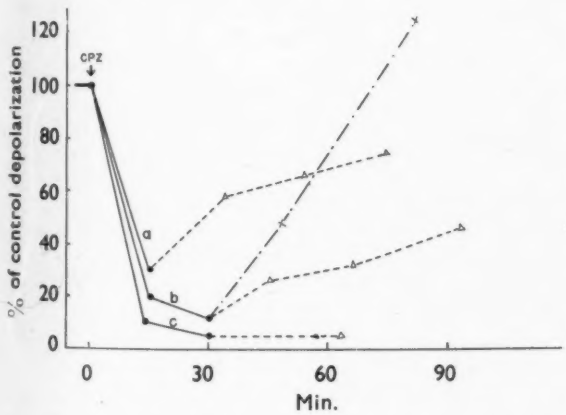

FIG. 9.—Toad, sartorius muscle. Magnitudes of depolarization caused by 8×10^{-6} g./ml. acetylcholine. **a**, 5 μM., **b**, 10 μM., **c**, 50 μM. chlorpromazine (●——●) given at zero time. Recovery in Ringer solution (Δ - - - Δ), and in chlorpromazine + neostigmine 10^{-6} g./ml. (X—.—X).

TABLE II

SYNTHESIS OF ACETYLCHOLINE BY THE GUINEA-PIG BRAIN

Figures refer to μg. of acetylcholine produced per mg. of acetone-dried powder.

Chlorpromazine Concentration (μM.)	Acetylcholine after Incubation Time of	
	1 hr.	2 hr.
0 (control)	0·18	0·34
1	0·18	0·32
10	0·19	0·33
100	0·18	0·32

DISCUSSION

Although Kopera and Armitage (1954), as well as Burn (1954), reported that the paralytic action of chlorpromazine in cats and rats was due to its direct action on muscle fibres, Ryall (1956) demonstrated that chlorpromazine inhibited neuromuscular transmission, with little effect on direct muscle stimulation, in the isolated phrenic nerve-diaphragm of rabbits. In our experiments using the nerve-muscle preparations of the frog sartorius and the rat diaphragm, chlorpromazine depressed the twitch responses to indirect stimulation much more markedly than those to direct stimulation. In addition, chlorpromazine decreased the size of the contractures of the frog rectus muscle due to acetylcholine while leaving those due to KCl unaffected. These facts indicate that the direct action of low concentrations of chlorpromazine on the muscle fibres is of minor importance. Since the conductivity of the nerve trunk was not affected by chlorpromazine, the main site of paralysis is probably the neuromuscular junction.

The blocking action is not of depolarizing type because the drug did not depolarize the endplates, did not cause fasciculation and did not antagonize the action of tubocurarine.

On the contrary, chlorpromazine reduced the endplate depolarization as well as the contracture caused by acetylcholine, and it was partly antagonized by anticholinesterases. In addition, it has been reported by many authors that chlorpromazine enhances the paralytic action of tubocurarine and its analogues (Wislicki, 1958). Our results show that the combined effect of chlorpromazine and tubocurarine should be classified as summation rather than as potentiation (" supra-additive synergism ") in the sense defined by Barstad (1956). It is therefore concluded

that the neuromuscular blocking action of chlorpromazine resembles that of tubocurarine in many respects.

There is evidence, however, that the action of chlorpromazine is not exactly like that of tubocurarine. First, it inhibits the muscle fibres directly and causes contracture when somewhat larger doses are applied: the mechanism of the latter action was not studied in the present work. Secondly, the anticurare agents exert only a slight antagonistic effect when the muscle is stimulated indirectly. Thirdly, post-tetanic potentiation of the muscle twitches is absent, and tetanus is well-sustained in the rat's diaphragm preparation. Fourthly, the dose response curves to acetylcholine on the frog's rectus abdominis muscle before and after treatment with chlorpromazine are not parallel, and they become bell-shaped after addition of anticurare agents.

Besides, whereas the enzymic synthesis of acetylcholine is unaffected, the release of acetylcholine by motor-nerve stimulation is inhibited by high concentrations of chlorpromazine. This inhibition may also constitute a part of the cause of neuromuscular block, indicating the multiplicity of the mode of action of chlorpromazine.

Moreover, the paralysis due to chlorpromazine could not be removed by repeated washing of the muscle preparations, except for the sartorius muscles of frogs and toads which recovered slowly. The irreversible nature of the paralysis has also been encountered in muscles of cats (Kopera and Armitage, 1954 ; Wislicki, 1958). Thus, the motor endplates are blocked by chlorpromazine with an affinity much stronger than that of tubocurarine, although the degree of irreversibility may vary largely among different kinds of muscle and different animal species.

Finally, although our results indicate that chlorpromazine has a direct influence on neuromuscular transmission, they do not exclude the possible participation of a central action of the drug on muscle tone in the intact animal. On the contrary, the neuromuscular junction seems to be less sensitive than the central nervous system to the drug, judging from the relatively high concentration required for neuromuscular block *in vitro*. The same conclusion may be drawn from the observations of several authors (Ryall, 1956 ; Wislicki, 1958 ; Otubo, 1959) that inhibition of striated muscle was consistently obtained only after intra-arterial injections ; either negative or inconsistent results were obtained after intravenous injections of doses sufficient to act on the central nervous system.

We should like to thank Professor G. Brownlee and Dr. D. W. Straughan, of the Department of Pharmacology, King's College, London, for their kindness in showing the method for the estimation of acetylcholine release from the rat diaphragm preparation to one of us (C. Y. L.). We are also indebted to Dr. E. Bülbring, of the Department of Pharmacology, Oxford, for correcting our English manuscript.

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THE QUANTITATIVE ASSAY OF CORTICOTROPHIN USING RATS TREATED WITH HYDROCORTISONE ACETATE

BY

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An attempt has been made to modify the Sayers method for the assay of corticotrophin by substituting for hypophysectomy an injection of sufficient hydrocortisone acetate to suppress the release of endogenous pituitary corticotrophin in intact rats over the period of the assay. The results of forty such assays of subcutaneous corticotrophin seem to indicate that this procedure is about 1½ to 2 times as efficient as the original method using hypophysectomized rats. For a 2 + 2 point assay it was necessary to use a minimum of twenty-four to thirty-two rats.

For some years attempts have been made to modify the technique involving adrenal ascorbic acid depletion for the assay of corticotrophin (ACTH) (Sayers, Sayers and Woodbury, 1948) by substituting hypophysectomized rats with animals in which the release of endogenous corticotrophin is sufficiently blocked by the administration of corticosteroids prior to the assay.

The investigations of Ingle and Kendall (1937), Ingle, Higgins and Kendall (1938), Sayers and Sayers (1947), and Gray and Munson (1951) showed that several corticosteroids inhibit the release of pituitary corticotrophin as judged by changes in ascorbic acid content and weight of the adrenal glands. At the time there was some confusion concerning the relative inhibitory effects of cortisone and deoxycortone. Sayers and Sayers (1947) were the first to suggest that several crystalline cortical steroids, including cortisone, hydrocortisone and deoxycortone, were most effective in blocking release of corticotrophin from the pituitary. Casentini, De Poli, Hukovic and Martini (1957), in a comparative test of the relative potencies of various corticosteroid preparations, found that deoxycortone had about the same activity as hydrocortisone acetate in a test on unilaterally adrenalectomized rats (Abelson and Baron, 1952; Porter and Jones, 1956).

On the other hand, Fortier, Yrarrazaval and Selye (1951) showed that both cortisone and cortisone acetate were completely ineffective in preventing the release of pituitary corticotrophin in response to stress, and Moya and Selye (1948), Gershberg, Fry, Brobeck and Long (1950) and Hall, Finerty, Hall and Hess (1951) were unable to confirm that deoxycortone acetate had an inhibitory effect under similar conditions. Hodges (1953, 1954) found that rats were far more suitable for a quantitative assay after treatment with deoxycortone acetate than after cortisone acetate, but even larger doses of deoxycortone failed to give a satisfactory preparation. Recently Hodges and Vernikos (1958) found that prednisolone and hydrocortisone were far more effective in inhibiting corticotrophin release and suggested the use of these preparations instead of deoxycortone acetate (Hodges, 1955) for a modified ascorbic acid depletion technique not involving hypophysectomy.

For over two years intact rats pretreated with hydrocortisone acetate have been used in this laboratory for subcutaneous and intravenous Sayers (Munson modification) assays of various corticotrophin preparations with satisfactory results, and an analysis of forty such subcutaneous bio-assays is reported in this paper. It is essential that the total dose of hydrocortisone acetate should be sufficient to suppress the release of endogenous pituitary corticotrophin over the period of the assay and that the subsequent injection of the corticotrophin preparation should give an ascorbic acid depletion similar to that in hypophysectomized rats. The blocking effect of hydrocortisone acetate was investigated in some detail in intact rats with different doses and routes of administration, in particular studying the intensity and duration of effect and the optimum time interval between injection of hydrocortisone and corticotrophin. From the results of these preliminary experiments, which will be reported separately, a standard procedure has been adopted.

METHODS

Male albino rats, weighing about 150 g. from Wistar stock, were kept in a room at constant temperature between 24 and 25° for 2 to 3 weeks before the test and were fed on Aberdeen standard diet. Hydrocortisone acetate was injected intraperitoneally into groups of eight rats in a total dose of 12 mg./100 g. body weight given in two equal doses over two days, at 2 p.m. on the day before the assay and at 8 a.m. on the following morning. The corticotrophin standard and test preparations were injected subcutaneously 4 hr. later. The total dose was dissolved in 0.6 ml. of gelatin per 150 g. body weight and given in three equal doses at 12, 1 and 2 p.m. About 10 to 15 min. after the last injection of corticotrophin the rats were anaesthetized with urethane injected subcutaneously; at 3 p.m. (45 to 50 min. later) 3 hr. after the first injection of corticotrophin each pair of adrenals was dissected out, weighed on a torsion balance to the nearest 0.1 mg. and the ascorbic acid content determined, the final response being expressed as μg. of ascorbic acid per 100 mg. of adrenals.

Determination of Ascorbic Acid.—Each weighed pair of adrenals was homogenized in a tube containing 0.5 ml. of 2.5% metaphosphoric acid, freshly prepared. An extra 9.5 ml. of the acid was then added and the tubes allowed to stand at least 30 min. It was possible to keep the homogenized suspension in the refrigerator overnight providing that freshly prepared standard solutions of pure ascorbic acid were kept under similar conditions. The tubes were centrifuged at about 2,500 revs./min. for 5 min., and an 8 ml. aliquot of the clear supernatant was added to a test tube containing 7 ml. of 4.53% sodium acetate (adjusted to *p*H 7.0 with acetic acid) and 1 ml. of 0.03% 2,6-dichlorophenolindophenol. The resultant pink colour was measured within 30 sec. of mixing with a Spekker absorptiometer, using a 2-cm. cell and Ilford filter No. 604 giving maximum transmission at 520 mμ. A calibration graph was prepared for ascorbic acid in concentrations up to 200 μg. per 10 ml. of 2.5% metaphosphoric acid and the unknown values for test solutions interpolated.

Drugs, Solutions and Media

Intraperitoneally.—Hydrocortisone acetate suspension (Hydro-Adreson, Organon; 25 mg./ml.).

Subcutaneously.—Corticotrophin: the weighed quantities of standard corticotrophin and various test preparations were dissolved in minimum volumes of 0.01 N HCl and 17% gelatin was added to give the

TABLE I

INDIVIDUAL RESULTS OF A SUBCUTANEOUS 3+3 POINT ASSAY

Purified corticotrophin was used in doses of 5, 10 and 20 μg. against 0.15, 0.3 and 0.6 i.u. of purified Home Standard (60 i.u./mg.), dissolved in 17% gelatin.

Total Dose	Adrenal Weight (mg.)	Ascorbic Acid per		Total Dose	Adrenal Weight (mg.)	Ascorbic Acid per		Total Dose	Adrenal Weight (mg.)	Ascorbic Acid per	
		Two Adrenals (μg.)	100 mg. Adrenals (μg.)			Two Adrenals (μg.)	100 mg. Adrenals (μg.)			Two Adrenals (μg.)	100 mg. Adrenals (μg.)
0·15 i.u. Standard	24·8	91·5	369	0·3 i.u. Standard	24·4	83·5	342	0·6 i.u. Standard	25·5	69·5	273
	26·3	98·0	373		30·9	107·5	348		27·3	79·5	291
	28·6	135·0	472		26·2	98·5	376		29·4	77·5	264
	29·9	100·0	334		32·4	102·0	315		31·7	101·5	320
	28·1	129·5	461		26·8	97·0	362		26·9	94·5	351
	21·2	84·5	399		31·1	119·0	383		27·2	92·0	338
	26·0	90·5	348		30·5	97·0	318		30·3	96·0	317
	23·9	97·5	408		26·0	76·5	294		28·0	81·5	291
Mean	26·1	103·3	395·5		28·5	97·6	342·3		28·3	86·5	305·6
5 μg. Test	25·4	91·5	360	10 μg. Test	26·9	80·0	297	20 μg. Test	26·6	92·5	348
	22·5	91·0	404		21·7	75·5	348		21·2	64·0	302
	24·2	95·5	395		29·2	98·0	336		34·2	103·0	301
	33·0	142·0	430		25·1	97·0	386		25·0	84·0	336
	31·4	112·5	358		23·5	82·5	351		29·2	85·5	293
	29·4	118·0	401		36·9	101·5	275		28·0	93·5	334
	30·4	121·0	398		23·9	90·5	379		33·3	70·0	210
	24·0	86·0	358		25·0	96·0	384		26·3	82·5	314
Mean	27·5	107·2	388·0		26·5	90·1	344·5		28·0	84·4	304·8

highest required dose in 0.6 ml. Dilutions with gelatin were made to give half the concentration of the higher dose for a 2+2 point assay and half and one quarter for a 3+3 point assay. Suitable doses were found in the range 0.15, 0.3, 0.6 and 1.2 units per 0.6 ml./150 g. of body weight. Solutions of gelatin (Type A, American Agricultural Chemical Co.) were made according to U.S.P., XV, p. 1094.

Anaesthetic.—Urethane (British Drug Houses, Ltd., 25% in distilled water, 0.65 ml./100 g. of body weight).

Results

Table I gives the results and Table II the analysis of variance of a typical corticotrophin assay using hydrocortisone acetate blocked rats. From these results it was calculated that the test material contained 31.0 U/mg. with fiducial limits (P=0.05) 21.7–44.2 U/mg. The root error mean square, s=38.0, the slope, b=−143.8, and the index of precision, λ=0.264.

TABLE II
THE ANALYSIS OF VARIANCE

Source of Variation	Sum of Squares	d.f.	Mean Square
Difference between standard and test	50	1	50
Regression	59,944	1	59,944
Departure from parallelism	88	1	88
Curvature	277	1	277
Difference of curvature ..	110	1	110
Doses	60,469	5	
Error	60,641	42	1,443·8
Total ..	121,110	47	

Fig. 1 shows the frequency distributions of the root error mean square, the slope, and the index of precision $\lambda\left(=\frac{s}{b}\right)$ of the forty assays. The mean values were found to be $s=36.7\pm0.9$, $b=162.7\pm7.5$, and $\lambda=0.244\pm0.013$.

From the accumulated data of the forty assays, the values of the combined variance and combined slope were calculated and found to be $s^2=1{,}389.2$ and $b=153.1$. Using these values in the formula

$$V(M) = \frac{s^2}{b^2}\left[\frac{1}{N_S} + \frac{1}{N_T} + \frac{(M-\bar{x}_S+\bar{x}_T)^2}{\Sigma(x-\bar{x})^2}\right]$$

from which approximate fiducial limits may be calculated, Table III was constructed showing the effect of varying the number of rats per assay on the errors which might be expected in a 2+2 point and a 3+3 point symmetrical assay ($N_S=N_T=\frac{1}{2}N$). The minimum variance would occur when the doses of test have been so chosen that they match exactly the doses of standard, that is, when $\bar{y}_T=\bar{y}_S$. In this instance

$$M-\bar{x}_S+\bar{x}_T=0 \text{ and } V(M)=\frac{s^2}{b^2}\left[\frac{1}{N_S}+\frac{1}{N_T}\right].$$

For the purpose of presentation of the results an arbitrarily chosen "maximal" error was also calculated if the doses of standard and test had been badly matched but overlapping, that is, if (1) S1=T2, in a 2+2 point assay with doses S1, S2, T1, T2 and if (2) S1=T2 and S2=T3, in a 3+3 point assay with doses S1, S2, S3, T1, T2, T3. In both these examples $M-\bar{x}_S+\bar{x}_T\left(=\frac{\bar{y}_T-\bar{y}_S}{b}\right)$ $=0.3010$ and $V(M)=\frac{s^2}{b^2}\left[\frac{1}{N_S}+\frac{1}{N_T}+\frac{0{\cdot}3010^2}{\Sigma(x-\bar{x})^2}\right]$.

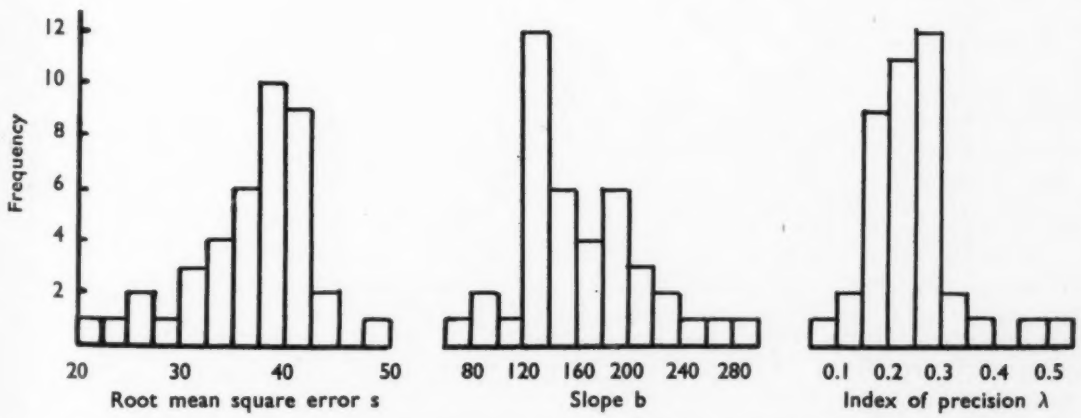

FIG. 1.—Histograms for root mean square error s (mean=36.7, median 38.1); slope b (mean=162.7, median=151.8) and index of precision λ (mean=0.244, median=0.228); n=40.

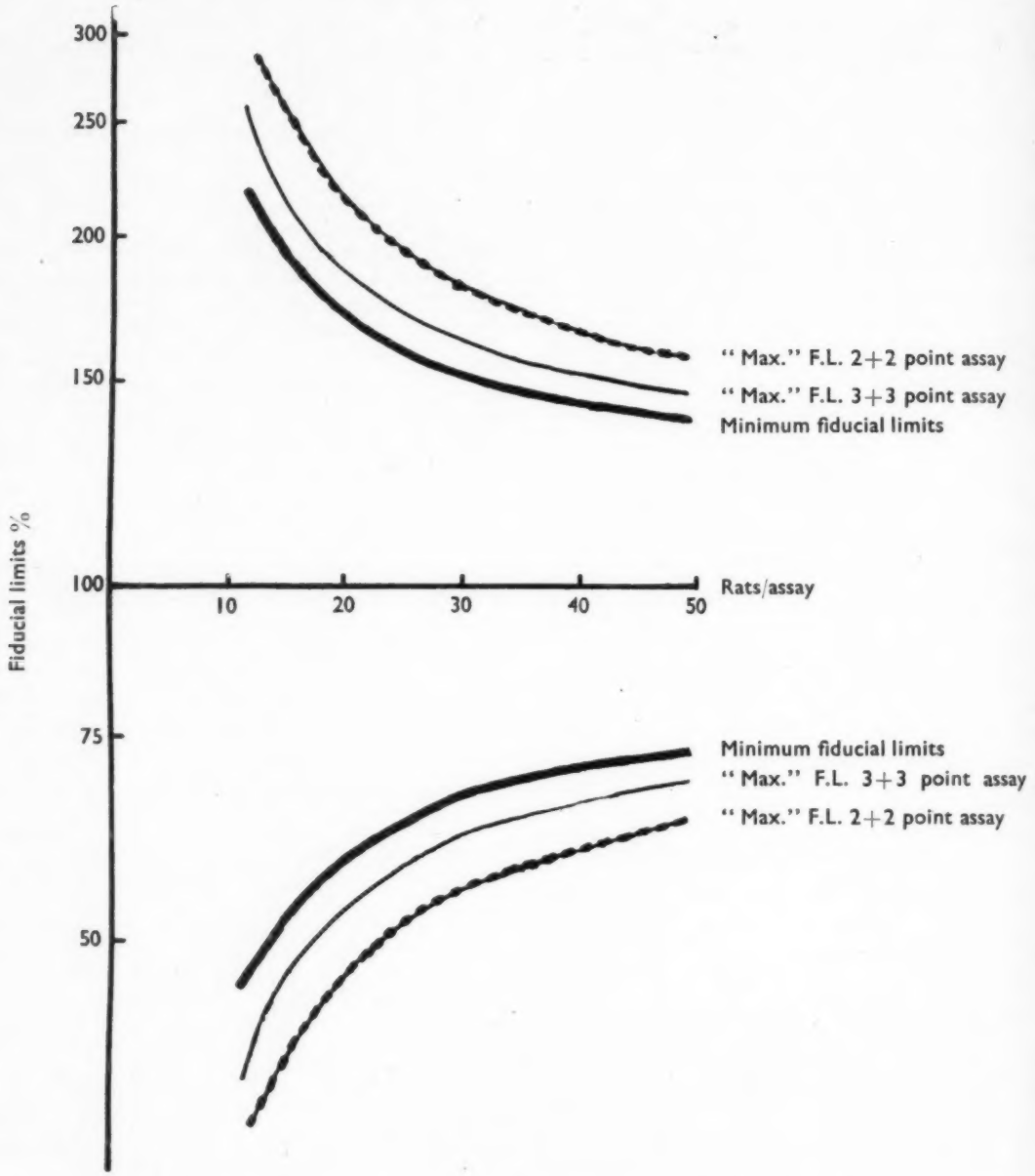

FIG. 2.—The effect of varying the number of rats per assay on the minimum and "maximum" fiducial limits ($P=0.05$). $s^2=1,389.2$; $b=153.1$.

TABLE III

THE EFFECT OF VARYING THE NUMBERS OF RATS PER ASSAY ON THE MINIMUM AND "MAXIMUM" FIDUCIAL LIMITS

$P=0.05$, $s^2=1,389.2$, $b=153.1$.

Rats/ Assay	2+2 pt. or 3+3 pt. Assay			2+2 pt. Assay			3+3 pt. Assay		
	Minimum Variance V(M) Min.	Weight W	Minimum Fiducial Limits	"Maximum" Variance V(M) Max.	Weight W	"Maximum" Fiducial Limits	"Maximum" Variance V(M) Max.	Weight W	"Maximum" Fiducial Limits
12	0·019756	51	47–212%	0·039511	25	35–288%	0·027162	37	39–254%
18	0·013170	76	57–177%				0·018109	55	51–197%
24	0·009878	101	62–161%	0·019756	51	51–197%	0·013582	74	57–176%
30	0·007902	127	66–152%				0·010866	92	61–164%
36	0·006585	152	68–146%	0·013170	66	58–172%	0·009055	110	64–156%
42	0·005644	177	71–142%				0·007761	129	66–151%
48	0·004939	202	72–139%	0·009878	101	63–159%	0·006791	147	68–147%

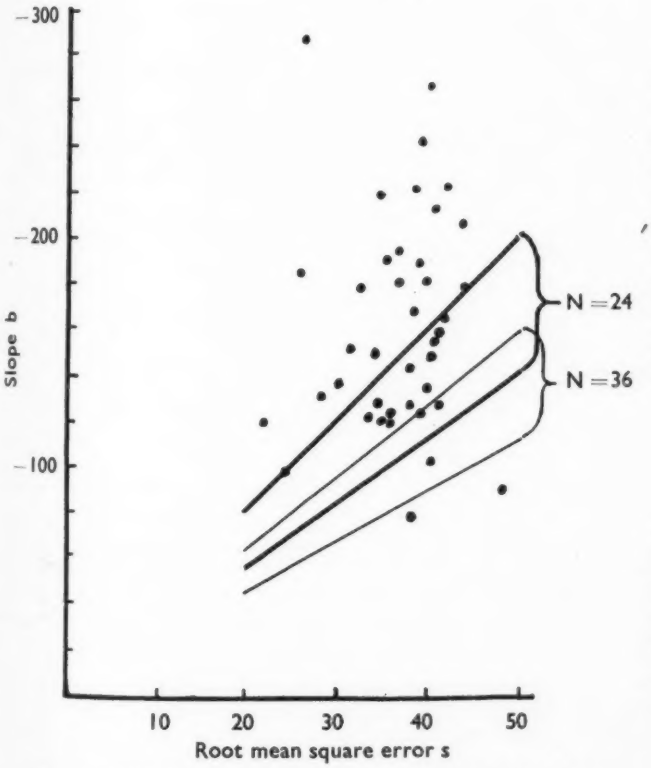

FIG. 3.—Values of s and b required to give fiducial limits of 50 to 200% when twenty-four or thirty-six rats per assay were used. ● represents individual values of s and b in forty assays of subcutaneous corticotrophin.

Fig. 2, which is constructed from the results in Table II, shows the number of rats required to give any desired degree of accuracy. Fig. 3 gives the values of s and b necessary to ensure the required fiducial limits of at the most 50 to 200% if twenty-four or thirty-six rats per assay are used. The individual values of s and b found in forty assays were plotted on the graph to indicate the number of assays where the desired precision would have been obtained using twenty-four or thirty-six rats per assay.

DISCUSSION

The results of forty assays of subcutaneous corticotrophin have been analysed by the method involving depletion of adrenal ascorbic acid in intact rats pretreated with hydrocortisone acetate. The total weight for the assays (1,340 rats) was found to be 6,283.2, so that the mean weight per rat was 4.69. This figure compares favourably with the value of 2.57 per rat in similar assays using hypophysectomized rats (Mussett and Perry, 1956), indicating that this modification appears to be about 1.5 to 2 times as efficient as the original Sayers (Munson modification) technique.

If limits of the order of 50 to 200% are required it can be seen from Fig. 2 that these limits can be obtained with twenty-four rats provided that the values of s and b are representative. However, because of the variations from assay to assay, it has been found necessary to use a rather larger number of rats to ensure a 200% upper fiducial limit. When the upper fiducial limit is 200%, $\log \frac{200}{100} = 0.3010 = t\sqrt{V(M)}$. When the error is minimal $V(M) = \frac{s^2}{b^2}\left[\frac{1}{N_S} + \frac{1}{N_T}\right]$ and therefore $\frac{s^2}{b^2} = \left(\frac{0.3010}{t}\right)^2 \div \left(\frac{1}{N_S} + \frac{1}{N_T}\right)$. By substituting N_S, N_T, and the appropriate value of t ($P = 0.05$) it was calculated that using twenty-four rats per assay $\frac{s^2}{b^2} = 0.12446$, so that if $s = 20$ then $b = 56.7$, and if $s = 50$, $b = 141.7$. All values of s and b above the line joining these points will give fiducial limits of 50 to 200% or less when twenty-four rats per assay are used and the doses have been correctly chosen. Similarly if a "maximal" variance occurs then the corresponding points are $s = 20$, $b = 80.2$ and $s = 50$ and $b = 200.4$. When the forty values of s and b are plotted on the graph, twenty-two of the forty assays would have given the required precision if twenty-four rats per assay had been used, but the majority of the remainder lie between the two lines ($N = 24$) and may give fiducial limits greater than 50 to 200%. When the number of rats per assay is increased to thirty-six all but five assays lie above the upper line. In practice it had been found necessary to use thirty-two rats per assay.

We are most grateful to Professor J. H. Gaddum for his help and advice.

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Brit. J. Pharmacol. (1960), **15**, 101.

THE STRUCTURE-ACTIVITY RELATIONSHIPS OF THE ANTIVIRAL CHEMOTHERAPEUTIC ACTIVITY OF ISATIN β-THIOSEMICARBAZONE

BY

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As part of an investigation devoted to the development of new antiviral agents a compound of established antiviral activity has been subjected to systematic structural modification. The structure-activity data so obtained have been used in the design of new compounds, some of which are described. The compound chosen was isatin β-thiosemicarbazone, which has high activity against neurovaccinia infection in mice, and a 4-point parallel-line assay of *in vivo* chemotherapeutic activity has been developed, which has enabled the activity of the derivatives to be determined against isatin β-thiosemicarbazone as a standard. The overall dimensions of the isatin β-thiosemicarbazone molecule appear to be nearly maximal for the retention of high activity, as all substituents in the aromatic ring decrease the activity irrespective of their nature or position. The projection of the $-CS.NH_2$ group in relation to the ring nitrogen was found to be critical, as the α-thiosemicarbazone was inactive. A number of modifications of the side-chain were investigated: all led to reduction or loss of antiviral activity. The antiviral activity showed a positive correlation with chloroform solubility over a considerable range. The most active compound encountered was 1-ethylisatin β-thiosemicarbazone, with an activity of 286 (isatin β-thiosemicarbazone≡100). Isatin β-thiosemicarbazone showed no activity against 15 other viruses, and 20 related compounds showed no activity against ectromelia.

The antiviral effect of isatin β-thiosemicarbazone (I) against vaccinia virus was first observed by Thompson, Minton, Officer, and Hitchings (1953), who found that mice receiving a diet containing 0.6% of the compound were protected against intracerebral infection with about 100 LD50 of the virus. Their experiments were not designed to demonstrate the maximum effect of the compound, and it was subsequently shown (Bauer, 1955) that mice treated with isatin β-thiosemicarbazone by subcutaneous injection of 2 doses of 125 mg./kg. each day throughout the incubation period would survive intracerebral infection with about 10,000 LD50 of vaccinia virus without showing signs of illness. The compound also gave complete protection against 100 to 1,000 LD50 of virus when given in a single dose of 25 mg./kg. 18 hours after infection.

The activity of isatin β-thiosemicarbazone against vaccinia infection is thus fully equal to the effect of the sulphonamides and tetracyclines against the agents of the psittacosis-lymphogranuloma group. These organisms have long been described as viruses, but present opinion assigns them to the Rickettsiales, and isatin β-thiosemicarbazone thus remains the only true example of an antiviral agent active in experimental animals which confers a protection against infection which is sufficiently powerful to be of clinical interest.

It is therefore important to investigate the relationship between structure and antiviral activity, since the information gained, apart from its intrinsic importance in the chemotherapy of pox virus infections, may make it possible to establish principles governing activity which can be applied to other classes of antiviral agents yet to be discovered. The first steps in this direction were taken by Thompson *et al.* (1953), who considered that the antiviral activity required both the thiosemicarbazone side-chain and a cyclic component. In the present work the relationship of structure to activity has been studied in much greater detail. A number of related compounds

and derivatives of isatin β-thiosemicarbazone have been prepared and tested, the relationship between structure and activity has been fully elucidated, and some of the derivatives have been found to possess a therapeutic activity considerably greater than that of the parent compound.

METHODS

Synthesis of Compounds

The melting points and analytical data of new substituted isatin β-thiosemicarbazones prepared in the present investigation are given in Table I, together with literature references to the preparation of those isatins which are already known.

Ring-substituted Compounds.—These were prepared by refluxing equimolar quantities of thiosemicarbazide with the appropriate substituted isatin in 50% aqueous ethanol for several hours. The products which separated on cooling were collected and washed well with hot water. Crystallization from butanol afforded samples for analysis. *5-Carboxymethylisatin:* prepared from *p*-aminophenylacetic acid by the Sandmeyer synthesis, orange needles, m.p. 224°; found: C, 59.4; H, 3.5; N, 7.3; Calc. for $C_{10}H_7O_4N$: C, 59.9; H, 3.5; N, 7.0. *5-Ethoxycarbonylmethylisatin:* esterification of 5-carboxymethylisatin with ethanol and concentrated sulphuric acid gave 5-ethoxycarbonylmethylisatin as orange plates, m.p. 132°.

N-*substituted Compounds.*—*1-Pentylisatin:* equimolar quantities of 1-sodioisatin and pentyl bromide were heated under reflux for 48 hr. The mixture was filtered and the filtrate was extracted with a small volume of 2N sodium hydroxide, which on acidification with concentrated hydrochloric acid gave 1-pentylisatin as red plates, m.p. 47°. 1-Isopropyl- and 1-isobutylisatin were prepared similarly. *1-(2-Hydroxyethyl)-isatin:* equimolar quantities of 1-sodioisatin and 2-chloroethanol were heated under reflux for 48 hr. The mixture was filtered, the filtrate was reduced to small volume and the product separated on the addition of 60/80° petroleum ether. Crystallization from aqueous methanol gave orange needles, m.p. 118°. *5-Carboxymethyl-1-methylisatin:* methylation of 5-carboxymethylisatin with dimethyl sulphate in 2N sodium hydroxide gave 5-carboxymethyl-1-methylisatin as red needles, m.p. 180°. *5-Ethoxycarbonylmethyl-1-methylisatin:* esterification of 5-carboxymethyl-1-methylisatin with ethanol in concentrated sulphuric acid gave 5-ethoxycarbonylmethyl-1-methylisatin as bright orange needles, m.p. 143°. *1-Methyl-4-trifluoromethylisatin:* methylation of 4-trifluoromethylisatin (Sadler, 1956) with dimethyl sulphate in 2N sodium hydroxide gave 1-methyl-4-trifluoromethylisatin as large orange plates, m.p. 156°.

Compounds Substituted in the Side-chain.—*Isatin β-2-phenylthiosemicarbazone:* equimolar quantities of 2-phenylthiosemicarbazide (Mautner and Kumler, 1956) and isatin were heated under reflux in ethanol for 2 hr. The product was removed by filtration and washed with ethanol. Crystallization from butanol gave yellow plates, m.p. 210° (yield 80%). Found: C, 60.9; H, 4.2; S, 10.7; Calc. for $C_{15}H_{12}ON_4S$: C, 60.8; H, 4.1; S, 10.8. *1-Methylisatin β-2-phenylthiosemicarbazone:* equimolar quantities of 2-phenylthiosemicarbazide and 1-methylisatin were heated under reflux for 4 hr. in 50% aqueous ethanol. The ethanol was removed by distillation and the product which crystallized on cooling was recrystallized from 50% aqueous methanol: yellow needles, m.p. 175° dec. (yield 70%). Found: C, 61.9; H, 4.6; S, 10.3; Calc. for $C_{16}H_{14}ON_4S$: C, 61.7; H, 4.5; S, 10.3. The following compounds were prepared similarly. *Isatin β-4-methylthiosemicarbazone:* yellow needles from ethanol, m.p. 260° (decomp.). Found: C, 51.3; H, 4.4; N, 23.8; S, 13.4; Calc. for $C_{10}H_{10}ON_4S$: C, 51.3; H, 4.3; N, 23.9; S, 13.7. *Isatin β-4-allylthiosemicarbazone:* yellow needles from ethanol, m.p. 211°. Found: C, 55.5; H, 4.7; N, 20.7; S, 11.9; Calc. for $C_{12}H_{12}ON_4S$: C, 55.3; H, 4.7; N, 21.5; S, 12.3. *Isatin β-4-phenylthiosemicarbazone:* yellow needles from ethanol, m.p. 255° (decomp.). Found: C, 60.9; H, 4.2; N, 18.6; S, 10.5; Calc. for $C_{15}H_{12}ON_4S$: C, 60.8; H, 4.1; N, 18.9; S, 10.8. *Isatin β-5-methylthiosemicarbazone:* yellow needles from ethanol, m.p. 257° (decomp.). Found: C, 51.3; H, 4.4; N, 23.5; S, 13.9. Calc. for $C_{10}H_{10}ON_4S$: C, 51.3; H, 4.3; N, 23.9; S, 13.7.

Miscellaneous Compounds.—*Isatin α-thiosemicarbazone* (prepared by Dr. C. G. Raison). Hot solutions of isatin α-anil (2.22 g. in 10 ml. alcohol) and thiosemicarbazide (0.91 g. in 10 ml. water) were mixed and boiled for 1 hr. When cold, the separated crystals were recrystallized from aqueous dimethylformamide. The compound formed brick-red crystals, m.p. 216-217° (decomp.). Found: C, 48.8; H, 3.8; S, 14.4. Calc. for $C_9H_8ON_4S$: C, 49.1; H, 3.7; S, 14.5. *3-Formyl-1-methyloxindole thiosemicarbazone:* equimolar quantities of 3-formyl-1-methyloxindole (Julian, Pikl, and Boggess, 1934) and thiosemicarbazide were refluxed in aqueous ethanol. The product, which separated on cooling, was recrystallized from aqueous ethanol, yellow plates, m.p. 219°. Found: C, 53.6; H, 4.5; S, 12.3; Calc. for $C_{11}H_{12}ON_4S$: C, 53.2; H, 4.8; S, 12.9. *1-Acetylindoxyl thiosemicarbazone:* equimolar quantities of 1-acetylindoxyl (Vorländer and Drescher, 1901) and thiosemicarbazide were refluxed in aqueous ethanol. The product separated from the hot reaction mixture and was recrystallized from a large volume of ethanol, white plates, m.p. 242°. Found: C, 53.2; H, 4.9; S, 12.9; Calc. for $C_{11}H_{12}ON_4S$: C, 53.2; H, 4.8; S, 12.9. *β-1-Benzimidazolylpropionthioamide:* a solution of 0.1 mole of β-1-benzimidazolylpropionitrile (Efros, 1953) in 20 ml. of 30% (w/v) ammoniacal ethanol was saturated with hydrogen sulphide and allowed to stand for 2 days. The ethanol was removed under reduced pressure and the residue was crystallized several times from hot water giving white needles, m.p. 151° (yield 25%). Found: C, 59.1; H, 5.3; S, 15.5. Calc. for $C_{10}H_{11}N_2S$: C, 58.5; H, 5.4; S, 15.6.

TABLE I

SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

Literature references are to the preparation of the corresponding isatins.

Substituent	Literature Reference	m.p.°	Formula	Required %			Found %		
				C	H	S	C	H	S
5-Fluoro ..	Holt and Sadler (1958)	275	$C_9H_7ON_4SF$	45·4	2·9	13·4	46·0	3·2	13·2
6-Fluoro ..	Sadler (1956)	257	$C_9H_7ON_4SF$	45·4	2·9	13·4	45·4	2·9	13·8
7-Fluoro ..	Holt and Sadler (1958)	263	$C_9H_7ON_4SF$	45·4	2·9	13·4	45·6	3·0	13·4
4-Chloro ..	Sadler (1956)	288	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·4	2·2	12·7
6-Chloro ..	,,	270	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·5	2·4	12·7
7-Chloro ..	Holt and Sadler (1958)	272	$C_9H_7ON_4SCl$	42·4	2·7	12·6	42·2	2·7	12·2
4-Bromo ..	Sadler (1956)	294	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·2	2·2	10·5
6-Bromo ..	,,	290	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·2	2·3	10·9
7-Bromo ..	Holt and Sadler (1958)	275	$C_9H_7ON_4SBr$	36·1	2·3	10·7	36·5	2·3	10·3
4-Iodo ..	Sadler (1956)	281	$C_9H_7ON_4SI$	31·2	2·0	9·2	32·0	2·3	9·7
5-Iodo ..	Borsche *et al.* (1924)	270	$C_9H_7ON_4SI$	31·2	2·0	9·2	31·5	2·2	9·0
6-Iodo ..	Sadler (1956)	275	$C_9H_7ON_4SI$	31·2	2·0	9·2	31·7	2·2	9·1
7-Iodo ..	Holt and Sadler (1958)	263	$C_9H_7ON_4SI$	31·2	2·0	9·2	30·7	1·6	9·0
6-Methyl ..	Sadler (1956)	270	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	51·1	4·4	13·5
7-Methyl ..	Holt and Sadler (1958)	275	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	51·1	4·4	13·6
1-Methyl ..	Hantzsch (1921)	245	$C_{10}H_{10}ON_4S$	51·3	4·3	13·7	50·5	4·5	13·7
1-Ethyl ..	Michaelis (1897)	201	$C_{11}H_{12}ON_4S$	53·2	4·8	12·9	53·1	5·1	12·8
1-Propyl ..	,,	193	$C_{12}H_{14}ON_4S$	54·9	5·4	12·2	55·1	5·1	12·1
1-Isopropyl ..	—	225	$C_{12}H_4ON_4S$	54·9	5·4	12·2	54·9	5·3	12·1
1-Pentyl ..	—	184	$C_{14}H_{18}ON_4S$	57·9	6·2	11·0	57·7	6·0	11·0
1-Hydroxymethyl	Reissert and Handeler (1924)	230	$C_{10}H_{10}ON_3S$	48·0	4·0	12·8	48·1	4·3	13·2
5-Amino ..	Giovannini and Portmann (1948)	>320	$C_9H_9ON_5S$	45·9	3·8	13·6	46·0	4·4	12·9
5-Hydroxy ..	,, ,,	284	$C_9H_8O_2N_4S$	45·8	3·4	13·5	45·5	3·4	13·4
1-Benzyl ..	Antrick (1885)	268	$C_{16}H_{14}ON_4S$	61·9	4·5	10·3	62·5	4·5	9·4
1-(2-Hydroxyethyl)	—	247	$C_{11}H_{12}O_2N_4S$	50·0	4·5	12·1	49·9	4·6	12·2
1-Carboxymethyl	Langenbeck (1928)		$C_{11}H_{10}O_3N_4S$	47·5	3·6	11·5	47·7	3·9	11·0
5-Carboxymethyl	—	273	$C_{11}H_{10}O_3N_4S$	47·5	3·6	11·5	47·1	3·6	11·1
1-Acetyl ..	Suida (1878)	244	$C_{11}H_{10}O_2N_4S$	50·4	3·8	12·2	50·8	4·1	12·3
1-Ethoxycarbonylmethyl	Ainley and Robinson (1934)	183	$C_{13}H_{14}O_3N_4S$	51·0	4·5	10·5	51·0	4·6	10·4
1-Diethoxycarbonylmethyl	,, ,,	200	$C_{16}H_{18}O_5N_4S$	50·8	4·8	8·5	50·6	4·4	8·4
1-(2-Cyanoethyl)	Carlo and Lindwall (1945)	234	$C_{12}H_{11}ON_5S$	52·7	4·0	11·7	52·6	4·0	11·8
4,5-Benz ..	Martinet (1919)	275	$C_{13}H_{10}ON_4S$	57·8	3·7	11·9	57·8	3·7	11·9
6,7-Benz ..	,,	280	$C_{13}H_{10}ON_4S$	57·8	3·7	11·9	57·3	3·7	11·7
5-Ethoxycarbonylmethyl	—	215	$C_{13}H_{14}O_3N_4S$	51·0	4·5	10·5	51·3	4·6	10·3
5-Carboxymethyl-1-methyl	—	264	$C_{12}H_{12}O_3N_4S$	49·3	4·1	10·9	49·6	3·8	10·5
5-Ethoxycarbonylmethyl-1-methyl	—	183	$C_{14}H_{16}O_3N_4S$	52·6	5·0	10·0	53·3	5·3	9·8
1-Methyl-4-trifluoromethyl	—	263	$C_{11}H_9ON_4SF_3$	43·7	3·0	10·6	44·6	3·1	10·7
5-Carboxy ..	Giovannini and Portmann (1948)	273	$C_{10}H_8O_3N_4S$	45·5	3·0	12·1			
7-Carboxy ..	,, ,,	296	$C_{10}H_8O_3N_4S$	45·5	3·0	12·1	45·3	2·9	12·2

Physical Investigations

Infra-red Absorption Spectra. — Spectra were determined by using a Perkin-Elmer 21 double-beam recording spectrometer fitted with a rock salt prism.

Water/lipid Partition Coefficients.—Solubilities in chloroform and partition coefficients of the compounds between chloroform and water were determined spectroscopically at 25° using matched quartz cells in a Hilger Uvispek.

Biological Methods

Viruses.—The IHD strain of neurotropic vaccinia virus adapted to intracerebral passage in mice was used. A stock suspension was prepared from an infected mouse brain homogenized in normal horse serum or tissue culture medium (10% bovine serum, 0.25% lactalbumin hydrolysate, 5% papain digest broth, 80% Earle solution) and mixed with 3 volumes of glycerol. The stock preparation was stored at −20°. Mice were inoculated intracerebrally with a suitable dilution of this preparation in tissue culture medium, and the time elapsing between infection and death was recorded to the nearest half-day. Animals which were alive and well at the end of 14 days were considered to have survived indefinitely.

The origin of the other neurotropic viruses used has been described previously (Bauer and Bradley, 1956); some were insufficiently stable as glycerol suspensions, and inoculations were made from intact brain tissue stored at −20° and homogenized at the time of use.

Treatment.—Most of the test compounds were only slightly soluble in water, and were made up as suspensions. The dose chosen was inoculated subcutaneously in a volume of 0.1 ml. twice daily for 5 days, the first dose being given 2 to 5 hr. after infection.

Preliminary Test of Antiviral Activity.—In previous work (Bauer, 1958) it has been shown that the mean reciprocal survival time of mice infected intracerebrally with neurovaccinia virus has a linear regression upon the logarithm of the dilution of virus used for infection, and that this relationship can be used as the basis of a test of chemotherapeutic activity, since treatment with an active compound will cause a significant reduction of the mean reciprocal survival time over a very wide range of virus doses. The compounds examined in the present work received a preliminary evaluation in a test of this nature, in which groups of 6 mice infected intracerebrally with about 1,000 LD50 of neurovaccinia virus were treated with doses of 125 mg./kg. and the survival times were compared with those of a control group of 6 mice which were similarly infected but left untreated. Compounds which gave no significant ($P>0.05$) reduction of the mean reciprocal survival time at this dose were considered to be inactive.

Determination of Dose-response Curves. — Compounds which showed activity in the preliminary test were examined further at lower doses. It was found that the mean reciprocal survival time gave a linear regression on the logarithm of dose of compound between the minimum dose producing an observable effect and the dose giving indefinite survival of all animals. The curve obtained with 1-propylisatin β-thiosemicarbazone is shown in Fig. 1, in which the response obtained at doses of 0.25, 0.5, and 2.5 mg./kg. is shown in comparison with the responses observed in the corresponding control groups.

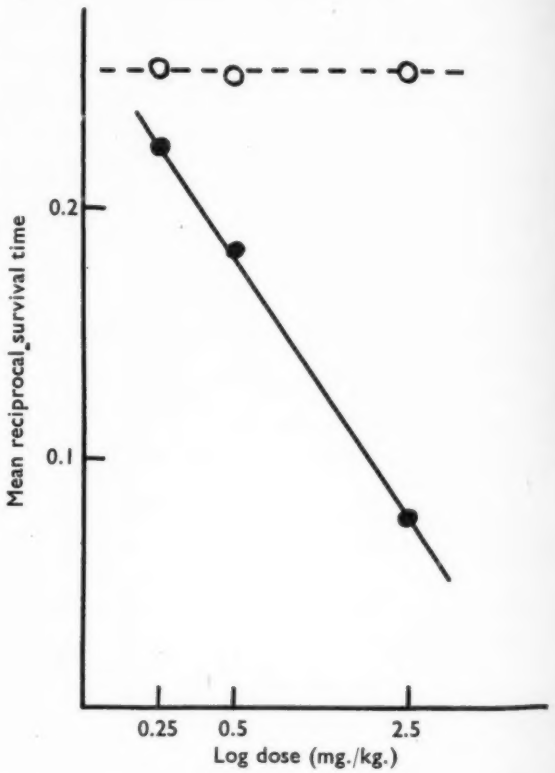


FIG. 1.—Dose-response curve of antiviral activity of 1-propylisatin β-thiosemicarbazone in mice infected intracerebrally with about 1,000 LD50 of neurotropic vaccinia virus. Ordinate, mean reciprocal survival time; abscissa, log dose of compound. Treated ●——●; controls, O— – —O.

Assay of Antiviral Activity.—The regression lines obtained for the dose-response curves clearly formed a suitable basis for an assay of antiviral activity based on the conventional 4-point design, in which the active compounds were assayed against isatin β-thiosemicarbazone, which was chosen as a standard. From the dose-response curve of a given compound 2 doses were selected, one being twice the other, which would give responses within the region of linearity when 1,000 LD50 of virus was used as the infecting dose. Two doses were similarly selected for isatin β-thiosemicarbazone; 1.25 and 2.5 mg./kg. were found to be suitable, although in some assays 1 and 2 mg./kg. were used. Four groups each of 6 mice were inoculated intracerebrally with about 1,000 LD50

of virus; 2 groups were treated with the test compound and 2 with isatin β-thiosemicarbazone, both in the selected doses. The survival times were converted to reciprocals (indefinite survival giving a reciprocal of zero) and the joint regression coefficient (b) was calculated for the 24 responses. The relative potency (R) was then obtained in the usual way as a function of the horizontal distance between the individual regression lines for test compound and standard from the relation

$$\log R = \log x_S - \log x_T - (\bar{y}_S - \bar{y}_T)\, b^{-1}$$

where x_S is the mean dose of isatin β-thiosemicarbazone, x_T is the mean dose of test compound, y_S is the mean response with isatin β-thiosemicarbazone and y_T the mean response with the test compound. The value obtained for R was then multiplied by 100 to give the activity based on isatin β-thiosemicarbazone taken as 100, and the result was finally multiplied by the ratio of the molecular weights of test compound and standard to give the activity expressed on an equimolar basis. The results of a typical assay are shown graphically in Fig. 2. The upper line represents the response obtained with doses of 1.25 and 2.5 mg./kg. of isatin β-thiosemicarbazone, and the lower line the responses with 5 and 10 mg./kg. of 7-methylisatin β-thiosemicarbazone; b was found to be −0.039, $\bar{y}_S$ 0.2176 and $\bar{y}_T$ 0.0747, giving a value of 0.89 for R, equivalent to a relative activity on an equimolecular basis of 94 for the 7-methyl derivative in comparison with the parent compound.

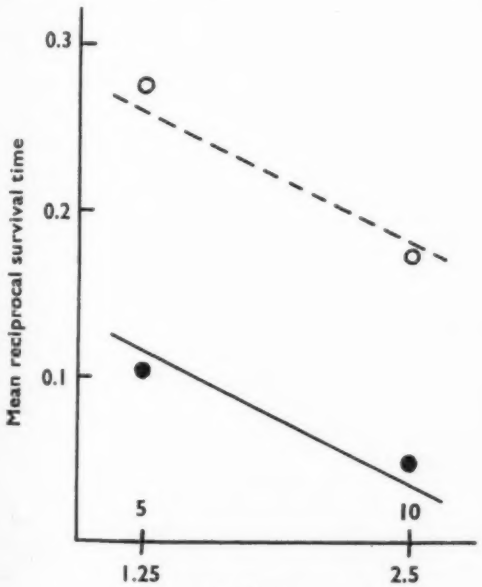


FIG. 2.—Four-point assay of antiviral activity of 7-methylisatin β-thiosemicarbazone in mice infected intracerebrally with about 1,000 LD50 of neurotropic vaccinia virus. Ordinate, mean reciprocal survival time; upper abscissa, log dose of test compound; lower abscissa, log dose of isatin β-thiosemicarbazone. ●——●, 7-methylisatin β-thiosemicarbazone; O— — —O, isatin β-thiosemicarbazone.

RESULTS

Effect on Antiviral Activity of Substitution in the Aromatic Ring

The introduction of substituents into the aromatic ring, as shown in Table II, usually results in reduction or total loss of activity. Substitution in the 5- position has a particularly marked effect, activity being lost except with substituents, such as fluorine, with small atomic or group radii. Activity is reduced to a lesser extent by substitution in the 4- and 6- positions, and some of the 7- substituted compounds still retain quite high activity.

An attempt was made to correlate the effect of substitution on antiviral activity with σ values, which are also given in Table II. The σ value, derived originally by Hammett (1949) from studies of the rates of hydrolysis of a series of substituted benzoic esters, affords a numerical measure of the

TABLE II

ANTIVIRAL ACTIVITY AND σ-VALUES OF RING SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

(No substituent≡100)

Substituent	4-Position	6-Position		5-Position	7-Position	$m\sigma$ Value for 5- and 7-Positions
	Activity	Activity	$p\sigma$ Value	Activity	Activity	
Amino ..				0		−0·161
Methyl ..	3·6	0·3	0·170	0	94	−0·069
Ethyl ..				0	~50	−0·043
Carboxymethyl ..				0		−0·027
Ethoxycarbonylmethyl ..				0		
Hydroxy ..				0		−0·002
Methoxy ..		0	−0·268	0·03		0·115
Fluoro ..		43·1	0·062	35·5	⩾20	0·337
Iodo ..	0	3·9	0·276	0	75·3	0·352
Carboxy ..				0	0	0·355
Chloro ..	9·9	11·7	0·227	4·2	98·3	0·373
Bromo ..	67·3	10·5	0·232	3·1	16	0·391
Trifluoromethyl ..					34·5	0·415
Nitro ..				0	0	0·710

effect of a substituent upon the reactivity of a side-chain in an aromatic system. The 5- and 7-substituents are listed together with the *meta* σ values, as both positions are *meta* to the β-carbon atom. The 6- substituents, which are *para* to the β-carbon atom, are given *para* σ values. No σ factors are quoted for the *ortho* substituents in the 4- position, as both steric and field effects are superimposed, to varying extents, on polar effects, making precise evaluation of these factors difficult (O'Sullivan and Sadler, 1957a). The 5- and 7-substituents are arranged in order of increasing *meta* σ values.

Table II shows that there is no parallel between antiviral activity and the σ values of the substituents. Considerable discrepancies occur between the data for the 5- and 7- substituents although their σ factors are identical, and, although a fairly wide range of σ values is covered in the 5- substituted series, ranging from the low value with the amino group through the intermediate values with the halogen substituents to the high value with the nitro group, most of these show zero activity. However, two generalizations emerge. Firstly, the smallest substituent (fluorine) in both the 5- and 6-positions decreases the activity the least (all other substituents quoted have much greater atomic or group radii), and secondly, substituents in the 7-position exert a noticeably smaller effect. This suggests that steric effects are far more important than the inductive and mesomeric effects of the substituents. Supporting evidence for this is that neither the 4,5- nor the 6,7-naphthisatin β-thiosemicarbazone possesses antiviral activity (see below). The observation that most activity is retained in the 7- substituted derivatives might be related to the relatively high solubility of the parent isatins. These compounds probably owe their enhanced solubilities to steric hindrance of the normal hydrogen-bonded dimer (O'Sullivan and Sadler, 1956), which suggests that the *N*-alkyl derivatives might have greater activity as these cannot dimerize in this fashion.

Effect of N-substitution upon Antiviral Activity

The activity of a number of *N*-substituted isatin β-thiosemicarbazones is shown in Table III. Alkylation in this position produces a marked rise in activity, reaching a maximum with 1-ethylisatin β-thiosemicarbazone, which with a relative activity of 286 is the most active compound so far encountered. Activity falls off as the chain is lengthened further, and the 1-pentyl derivative is practically inactive. High activity is retained in the 1-(2-hydroxyethyl) compound, but most of the other substituents which are either large or strongly polar have only residual or zero activity. This supports the hypothesis that isatin β-thiosemicarbazone should be monomeric and not form *inter*molecular bonds if high activity is to be retained.

TABLE III

ANTIVIRAL ACTIVITY OF *N*-SUBSTITUTED ISATIN β-THIOSEMICARBAZONES

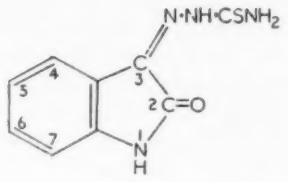
Substituent	Antiviral Activity
None	100
Methyl	202
Ethyl	286
Isopropyl	44
Propyl	28·5
Pentyl	3·4
Hydroxymethyl	42
1-Methyl-4-trifluoromethyl	48·4
2-Hydroxyethyl	204
Acetyl	87
Ethoxycarbonylmethyl	0
Diethoxycarbonylmethyl	0
2-Cyanoethyl	0
Carboxymethyl	0·02
5-Ethoxycarbonylmethyl-1-methyl	0
5-Carboxymethyl-1-methyl	0

Effect of Modifications in the Pyrrolidine Ring

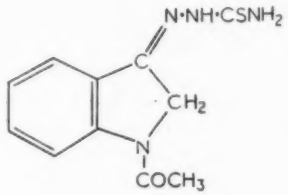
Modifications were next made at the 2- and 3-positions. The inactivity of 1-acetylindoxyl thiosemicarbazone (II) readily demonstrated that the α-carbonyl group was essential. Extension of the side-chain at the 3- position resulted in loss of activity, as 3-formyl-1-methyloxindole thiosemicarbazone (III) and 3-formylindole thiosemicarbazone were completely inactive. These and other similar results suggest that isatin β-thiosemicarbazone exerts its antiviral activity in the form of a six-membered, resonance-stabilized, hydrogen-bonded ring (IV), a type of bonding which has previously been demonstrated in the isatin β-oximes (O'Sullivan and Sadler, 1957b). Support for this hypothesis is provided by an analysis of the infra-red absorption data of isatin β-thiosemicarbazone and relevant reference compounds both in the solid state and in weakly polar solvents; however, the stability of such *intra*molecular bonds in an aqueous medium is uncertain. Isatin α-thiosemicarbazone may possess a similar *intra*molecular bonded structure (V), but it had no antiviral activity. This suggests not only that an all-planar configuration is

necessary but also that the orientation of the $-CS.NH_2$ group relative to the ring nitrogen is critical.

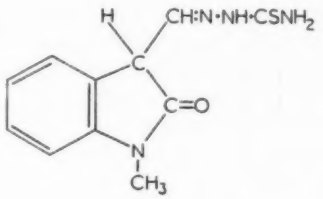
(I)



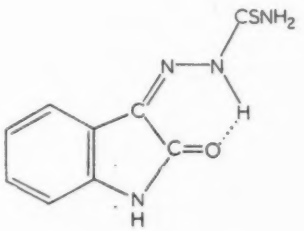
(II)



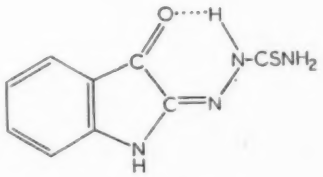
(III)



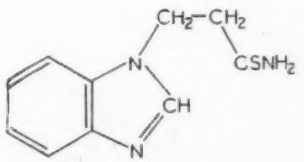
(IV)



(V)



(VI)



Effect of Substitution in the Side-chain

Finally, modifications were made to the thiosemicarbazone moiety, and the effects on antiviral activity are shown in Table IV. If *intra*molecular hydrogen bonding is of importance then replacement of the hydrogen atom on the nitrogen atom at the 2- position of the side-chain by another atom or group should result in loss of activity, as the molecule may no longer be held in configuration IV. Reference to Table IV shows this to be the case. The rest of the results presented indicate that an unsubstituted $-CS.NH_2$ group is essential for retention of activity.

TABLE IV

EFFECT OF SUBSTITUTION IN THE SIDE-CHAIN ON THE ANTIVIRAL ACTIVITY OF ISATIN β-THIOSEMICARBAZONE

R′–	R″=	R‴–	Antiviral Activity
H–	S=	H–	100
H–	O=	H–	0
H–	S=	C_6H_5	0
H–	HN=	H–	0
H–	S=	CH_3–	0
C_6H_5	S=	CH_3–	?
C_6H_5	S=	H–	0
H–	S=	CH_2=CH–CH_2–	4

R′–	R″–	R‴–	Antiviral Activity
—	CH_3–S–	H–	0

Miscellaneous Compounds

The following compounds were inactive: isatin, thiosemicarbazide, isatin β-amidinohydrazone, isatin β-hydrazone, di-β-isatinazine, 3-formyl-1-methyloxindole thiosemicarbazone, 1-acetylindoxyl thiosemicarbazone, α-naphthisatin β-thiosemicarbazone, β-naphthisatin β-thiosemicarbazone, isatin β-semicarbazone, isatin α-thiosemicarbazone, β-1-benzimidazolylpropionthioamide.

Activity of Isatin β-Thiosemicarbazone Against Other Viruses

In tests carried out in mice by the same method as the preliminary test of activity against neurovaccinia virus, isatin β-thiosemicarbazone in repeated doses of 100–125 mg./kg. had no therapeutic effect upon intracerebral infection with

poliomyelitis, influenza (NWS strain), rabies (Flury strain), Ilhéus, Wyeomyia, Zika, California, pseudolymphocytic choriomeningitis (Sandom strain, identical with ectromelia), Ntaya, Semliki, herpes, dengue 1, Anopheles A, Anopheles B or MM viruses.

Activity of Substituted Isatin β-Thiosemicarbazones and Related Compounds Against Ectromelia

In view of the close antigenic relationship between vaccinia and ectromelia viruses many of the compounds were tested against intracerebral infection with ectromelia (pseudolymphocytic choriomeningitis) virus. The following compounds showed no detectable activity when tested at doses ranging in some cases up to 100 mg./kg.: isatin β-semicarbazone, isatin β-4-phenyl thiosemicarbazone, isatin β-amidinohydrazone, isatin β-hydrazone, di-β-isatinazine, 3-formyl-indole thiosemicarbazone, and the β-thiosemicarbazones of the following substituted isatins: 5-fluoro-, 5-chloro-, 5-bromo-, 5-methoxy-, 5-ethyl-, 5-nitro-, 4-methyl-, 7-chloro-, 7-nitro-, 1-methyl-, 1-hydroxymethyl-, 1-(2-hydroxyethyl)- and 1-(2-cyanoethyl)-isatin.

Discussion

Isatin is a diketonic compound and it has many of the properties of a simple diketone. A number of dicarbonyl compounds have been reported to possess antiviral activity against Newcastle disease virus and the PR8 strain of influenza virus in fertile eggs (McLimans, Underwood, Slater, Davis, and Siem, 1957), but they were also virucidal *in vitro* and the effect was presumably due to the degradation of the amino-acids of the virus by the Strecker reaction. A mechanism of this kind cannot explain the activity of isatin β-thiosemicarbazone, since isatin has no activity against vaccinia virus *in vivo*, and the antiviral activities of the substituted isatin β-thiosemicarbazones show no correlation with the dehydrogenase activity of the corresponding isatins, as shown in Table V. The logarithms of the chloroform solubilities of the compounds shown in Table V show a positive correlation with antiviral activity (correlation coefficient 0.775, $P<0.01>0.001$). The increased activity of the simple *N*-alkyl derivatives may therefore result entirely from their improved lipid solubility, which might more readily enable them

Table V

σ-VALUES AND DEHYDROGENASE ACTIVITIES OF SUBSTITUTED ISATINS, WITH ANTIVIRAL ACTIVITIES AND PARTITION COEFFICIENTS OF THE CORRESPONDING β-THIOSEMICARBAZONES

Figures in column three are obtained from the reciprocals of the times required for the systems to decolorize methylene blue in 10^{-4} M solution of the substituted isatin and 0·05 M DL-alanine (O'Sullivan and Sadler, 1957c).

Substituent	σ	Dehydrogenase Activity Relative to DL-Alanine	Antiviral Activity	Solubility in Chloroform (mg./100 ml.)	Chloroform Water Partition
4-Methyl	—	11	3·4	8	0·063
6-Methyl	−0·170	30	0·3	18	0·15
7-Methyl	−0·069	55	88·8	16	0·185
None	0·0	100	100	32	0·33
6-Fluoro	0·062	50	39·8	16	0·48
5-Methoxy	0·115	18	0·03	3	0·077
4-Chloro	—	105	8·6	10	0·34
6-Chloro	0·227	180	4	21	0·69
4-Bromo	—	200	49·5	10	0·205
5-Fluoro	0·337	—	35·5	4	—
7-Carboxy	0·355	—	0	0	∞
7-Chloro	0·372	210	85	29	0·097
1-Methyl	—	—	190	160	0·083
1-Acetyl	—	—	87	255	—
1-Pentyl	—	—	0	>200	0·0
1-Diethoxycarbonylmethyl	—	—	0	>200	0·33
1-Ethyl	—	—	286	2,170	—
1-(2-Hydroxyethyl)	—	—	204	25	—
1-Butyl	—	—	28·5	1,600	—
1-Isobutyl	—	—	44	2,100	—

to pass the blood-brain barrier and so reach the neurotropic strain of vaccinia used as the test virus. This would also account for the inactivity of the compounds bearing strongly polar substituents. The reduction in activity produced by substitution in the aromatic ring suggests that steric factors are of importance, in that this region of the molecule must not exceed certain dimensions if activity is to be retained.

The general configuration of the isatin molecule resembles that of the purines and also tryptophan and its derivatives, but there is no evidence available as to whether isatin β-thiosemicarbazone acts as an antimetabolite to these compounds; in particular, an action against 5-hydroxytryptamine seems to be excluded by the lack of antiviral activity of 5-hydroxyisatin β-thiosemicarbazone.

The requirement for sulphur in the side-chain suggests that metal chelation may be of importance for antiviral activity, particularly of copper, and it is of interest to note in this connexion that vaccinia virus contains copper (Hoagland, Ward, Smadel, and Rivers, 1941), a circumstance which might enable it to take up isatin β-thiosemicarbazone selectively, and that salts of copper have a therapeutic effect against neurovaccinia infection in mice (Bauer, 1958). However, even if chelation is concerned, the configuration of the ligand is also critical, since, whereas all thiosemicarbazones chelate copper, only a very limited number have antivaccinial activity.

Several possibilities exist for the *in vivo* conversion of the compound into biologically more active substances. For instance, simpler substituted thioureas may be obtained, which bear resemblance to some of the phenylthiourea derivatives recently described by Doub *et al.* (1958). These compounds have marked tuberculostatic activity (Youmans *et al.*, 1958), but this is of little import as antiviral and tuberculostatic activities rarely run parallel (Hurst and Hull, 1956); benzthiouracils could also be obtained, and substituted phenoxythiouracils have considerable antiviral activity (Bauer, 1955), but this route is rendered unlikely as the suggested mechanism for the formation of the benzthiouracil seems chemically improbable, although it might occur biologically. Formation of a tricyclic compound by loss of the elements of water from the α-carbonyl group and the terminal amino group of isatin β-thiosemicarbazone is possible, and it has been clearly demonstrated that both these groups are essential for the retention of activity. However, the resulting structure is not sufficiently close to growth inhibitors such as 2-thiodenine or 6-thiopurine (Elion *et al.*, 1953) to be considered as a purine antagonist. The relationship to thioalloxazine is also somewhat remote, but the enhanced activity which results from 2-hydroxyethyl substitution in the 1-position (Table III) makes the synthesis of related glycosides of considerable interest as potential riboflavine antagonists.

The structures of these compounds seem too remote from that of nicotinamide to be considered as competitive inhibitors; some support is given by the fact that similar arguments have been used in the case of the tuberculostatic compound isoniazid (isonicotinic acid hydrazide) (Zatman *et al.*, 1953). Obviously other heteroaromatic thioamides containing more than one nitrogen atom per heterocycle are worthy of investigation, but it would seem to be essential for the thiocarbamyl group to be attached directly to one of the rings, as β-1-benzimidazolylpropionthioamide (VI) is inactive. The lack of activity of isatin β-thiosemicarbazone against other viruses, particularly ectromelia, is difficult to explain in accordance with the commonly held view that viruses control the metabolism of the cell in which they are multiplying, for this implies that the final common path in virus multiplication must be the same for all viruses. A possible explanation is that isatin β-thiosemicarbazone chelates with the copper present in vaccinia virus and thereby exerts a detrimental effect upon virus multiplication; if this is the case, then the lack of activity against other viruses implies that they do not contain copper, or, if they do, then the copper is either inaccessible or held in a chelate of higher stability constant.

We wish to thank Miss M. E. Pitman for her assistance in the preparation of the compounds.

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PHARMACOLOGICAL PROPERTIES OF THALIDOMIDE (α-PHTHALIMIDO GLUTARIMIDE), A NEW SEDATIVE HYPNOTIC DRUG

BY

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Thalidomide (α-phthalimidoglutarimide, " Distaval," " Contergan ") is a new sedative hypnotic drug which produces no toxic effects when administered orally to animals in massive doses. This lack of toxicity may be due to a limited absorption. The drug has a quietening effect on the central nervous system, reducing the voluntary activity of laboratory animals and promoting sleep. Unlike the barbiturate drugs it does not cause an initial excitation in mice, incoordination or narcosis. It potentiates the actions of other central nervous system depressants, in particular the barbiturates. Its sedative effects are counteracted by central nervous system stimulants. It has no deleterious side effects and does not affect the heart, respiration or autonomic nervous system.

Thalidomide is a derivative of glutamic acid, its chemical structure being shown in Fig. 1.

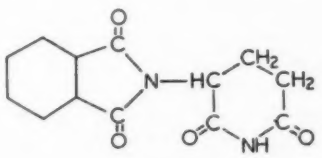

FIG. 1

It was first synthesized by Kunz, Keller, and Muckter (1956), who also described its main pharmacological properties. It occurs as a pure white, tasteless crystalline powder with a melting point of 271°. The substance is sparingly soluble in water, methanol, ethanol, acetone, and glacial acetic acid but readily soluble in dioxane, dimethyl formamide, pyridine and chloroform. It is insoluble in ether and benzene. Chemically it is related to bemegride (α-ethyl-α-methyl-glutarimide) and to glutethimide (β-ethyl-β-phenyl-glutarimide), but its pharmacological properties are different.

METHODS

Except where specifically stated thalidomide was used as a suspension in a 1% solution of carboxymethylcellulose.

Acute Toxicity

The acute toxicity of thalidomide was determined by the oral, subcutaneous and intraperitoneal routes in male albino mice weighing between 19 and 21 g. Comparisons were made with phenobarbitone sodium and glutethimide. Where possible the regression of percentage mortality as probits on the logarithm of the dose was found, and the LD50s and limits of error ($P=0.95$) were calculated by the method of Litchfield and Wilcoxon (1949).

Subacute Toxicity

The subacute toxicity of thalidomide was determined in young rats which were dosed orally each day over four weeks. Their body weights were recorded twice weekly and at the end of the period the rats were killed and a post-mortem carried out. Microscopic preparations of the major organs were also examined.

The pharmacological actions of thalidomide on the central and autonomic nervous systems and on the cardiovascular system were studied in mice, rats and anaesthetized cats, according to the following methods.

Central Nervous System

Motor Activity.—The sedative effects of thalidomide were determined on the voluntary motor activity of mice by the photobeam method of Dews (1953). The apparatus was a perspex tank measuring 15 in. × 9 in. A beam of light shone across the short axis on to a phototransistor (Mullard OCP71). Interruption of the light beam activated a Thorp impulse counter (C. F. Palmer) and a magnetic digital counter. Groups of ten mice were placed in the tank 30 min. after dosing

and the counts recorded at 15 min. intervals over 45 min. For the calculations the counts between 15 and 45 min. were used, since the initial count was affected by the exploratory hyperactivity of the mice. A control group was always run concurrently with a treated group and the response of the treated group calculated as a percentage of the control value.

Narcotic Activity.—This was tested in mice by placing them on their backs and recording the number which had lost their righting reflex.

Movement Co-ordination and Holding Reflex.—The effects of thalidomide on motor co-ordination were compared with phenobarbitone and glutethimide by the rotating rod method of Gross, Tripod and Meier (1955). Groups of ten mice were treated with the respective drugs and placed at 30 min. intervals on to a wooden rod rotating at 15 revs./min. The times the individual mice were able to remain on the rod were measured and the average determined for each group. Mice remaining on the rod for more than 2 min. were regarded as unaffected.

Depression of the holding reflex was assessed by suspending mice by the front legs from a horizontal wire and observing their ability to bring up their back legs.

Anticonvulsant Activity.—This was determined by measuring the ability of thalidomide to protect mice against the convulsant actions of leptazol and strychnine.

Analgesic Activity.—Thalidomide was tested for analgesic activity in mice and rats by the hot plate method of Woolfe and Macdonald (1944), the tail pinching method as described by Bianchi and Francheschini (1954) and electrical stimulation of the feet (Dodds, Lawson, Simpson and Williams, 1945) and tail (Kraushaar, 1953). It was also tested for its ability to potentiate the analgesic actions of morphine and pethidine.

Hypothermic and Antipyretic Activities. — These were tested in normal rabbits and in rabbits made febrile by an injection of a suspension of inactivated *Escherichia coli* bacteria.

Autonomic Nervous System, Heart and Respiration

The pharmacological effects of thalidomide on the autonomic nervous system were studied in cats anaesthetized with chloralose. Blood pressure was recorded from the left carotid artery and respiration from a tracheal cannula by the method described by Paton (1949). The right preganglionic cervical sympathetic nerve was severed and the rostral stump stimulated with rectangular pulses of approximately 8 V and of 2.5 msec. duration for 5 sec. An electrocardiograph was taken from lead II using subcutaneously implanted needle electrodes. The record was made with a Cambridge direct-writing cardiograph. Thalidomide, because of its low water solubility, could not be given intravenously. It was, therefore, injected into the peritoneal cavity or directly into the duodenum.

Rabbit isolated hearts were perfused with Ringer-Locke solution containing 10 mg./l. of thalidomide and records were made of the heart beat and coronary flow.

Gastro-intestinal Tract

The effects of thalidomide on intestinal muscle were studied *in vitro* on the isolated duodenum of the rabbit and the isolated ileum of the guinea-pig. *In vivo* the charcoal meal test was used as described by Bryant, Felton and Krantz (1957).

Urinary System

Kidney effects of thalidomide were studied in rats hydrated with an oral dose of water (50 ml./kg.) and placed into individual metabolism cages.

Interactions with Other Drugs

Barbiturates.—The duration of hexobarbitone anaesthesia (125 mg./kg., intraperitoneally) was compared between groups of control and thalidomide treated mice.

Alcohol.—Possible potentiation of the toxic effects of alcohol was studied by determining the LD50s of alcohol in groups of mice given different doses of thalidomide.

Reserpine and Chlorpromazine. — These were studied in two ways. Firstly a comparison was made of the duration of catatonia in groups of mice given these drugs with and without thalidomide. Secondly the effects of thalidomide on the potentiating action of these drugs on hexobarbitone were determined as described by Kopmann and Hughes (1958).

Methylamphetamine and Methylphenidate.—These stimulant drugs were administered to mice previously treated with thalidomide and the effects on motor activity observed. The LD50s of these stimulant drugs were also determined in mice treated with thalidomide and compared with the values for normal mice.

RESULTS

Toxicity

Acute Toxicity.—Thalidomide proved to be virtually non-toxic. As reported by Kunz *et al.* (1956), mice tolerated the maximum oral dose (5 g./kg.) that could be administered, without ill effects. They showed sedation without a preliminary excitation. Motility was considerably reduced, the mice bunching in a corner of the cage and going to sleep. There was no incoordination in their movements, and narcosis, catalepsy, and convulsions were absent. Sleeping mice could be easily aroused, and then they resumed normal but slow movements and showed little interest in their surroundings. In contrast both phenobarbitone and glutethimide caused initial excitation, marked ataxia, and incoordination of movement followed by narcosis and death. The LD50s of these two drugs are compared with thalidomide in Table I.

TABLE I

THE ACUTE ORAL TOXICITIES OF THALIDOMIDE, GLUTETHIMIDE AND PHENOBARBITONE IN MICE

Compound	LD50 (g./kg.)	Confidence Limits (P=0·95; g./kg.)
Thalidomide	>5	—
Glutethimide	0·52	0·46–0·58
Phenobarbitone sodium ..	0·27	0·25–0·29

Mice survived intraperitoneal doses as high as 4 g./kg. Guinea-pigs given 650 mg./kg. orally became quiet and sedated, while 400 mg./kg. injected intramuscularly produced no effects at all, insoluble material remaining unabsorbed at the site of the injection for over two days.

The low toxicity of thalidomide has been observed in man. Twenty cases of accidental or intentional overdosage have been reported (de Souza, 1959; Burley, personal communication), and all recovered uneventfully. It may well be that the absence of toxicity is due to a limited absorption, for the compound has a low solubility in body fluids, and when administered parenterally remains at the site of the injection. In the absence of a suitable assay method absorption studies have not yet been made.

Subacute Toxicity.—Two groups of ten rats given daily oral doses of 250 mg. and 1,000 mg./kg. respectively over a period of 21 days grew normally compared with untreated controls (Table II). Blood examinations showed no abnormal changes in the red and white cell counts.

TABLE II

THE EFFECT OF REPEATED ADMINISTRATION OF THALIDOMIDE ON THE GROWTH OF MALE RATS

Treatment	Daily Dose (mg./kg.)	Average Body Weights (in g.) at Stated Times from Beginning of Experiment			
		0 Days	7 Days	14 Days	21 Days
Controls ..	0	135	177	207	228
Thalidomide	250	142	179	214	226
,,	1,000	138	171	197	226

There were no abnormal constituents in the urine. When the rats were killed no significant pathological changes were seen in the major organs or glands. Histological sections of the thyroid glands showed only a slight reduction in the colloid material, suggesting a slight depression of secretory activity. Murdoch and Campbell (1958) reported that thalidomide depressed the uptake of radioactive iodine by the thyroid in humans, but this has been shown to be common to tranquillizing drugs (Friedell, 1958). Greene and Farran (1958) stated that the effects on the human thyroid were negligible. The thyroid weights of our rats were normal, and histological changes in the glandular epithelium which one normally associates with an antithyroid drug were absent. A slight depression of glandular activity can be expected from a sedative drug due to a decreased demand for the thyroid hormone following the reduction in body activity.

Central Nervous System

Motor Activity.—Thalidomide caused a marked reduction in motor activity. The results obtained, using eight groups of mice at each dose level, are shown in Fig. 2. It proved impossible to compare the activity of thalidomide with phenobarbitone and glutethimide by this method, as mice varied considerably in their response to the latter drugs between hypermotility due to excitation and lack of movement due to narcosis. Kunz *et al.* (1956) using activity cages found the oral doses inducing sleep in mice to be 100 mg. for thalidomide, 40 mg. for phenobarbitone sodium, and 75 mg. for glutethimide, all per kg. body weight.

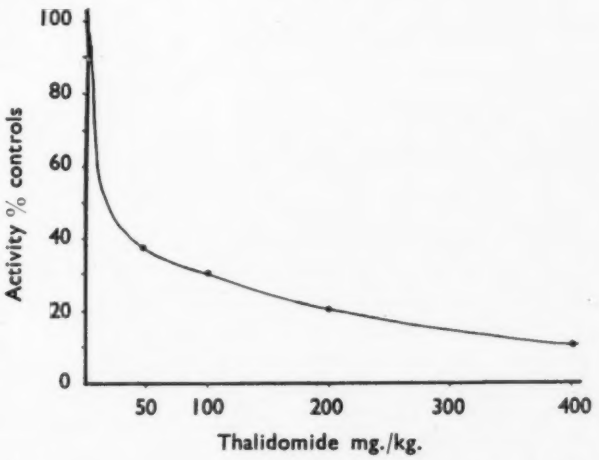

FIG. 2.—The effect of thalidomide on voluntary motor activity in mice. Each point is the mean of eight groups.

Narcotic Activity.—Thalidomide was devoid of a narcotic action. Even in maximum doses (5 g./kg.) it did not affect the righting reflex in mice. It behaved quite differently in this respect from glutethimide and phenobarbitone, which both had a strong narcotic effect.

Movement Co-ordination and Holding Reflex.—Even in very large doses thalidomide showed no effect on motor co-ordination. Mice given oral doses of 5 g./kg. were able to stay on the rotating rod for more than 2 min. With phenobarbitone and glutethimide the mice were unable to maintain their locomotion and balance and fell off the rod.

Anticonvulsant Activity.—Thalidomide did not antagonize leptazol-induced convulsions in mice, even in very large doses (1.6 g./kg.). Against spinal convulsions induced by strychnine there was a slight protection, probably due to the sedative action of thalidomide in reducing sensory stimuli. Similar results were obtained by Fincato (1957). Kunz *et al.* (1956) found it ineffective against electroshock seizures in the rat.

Analgesic Activity.—Thalidomide showed no analgesic activity in the tests described. In this respect it behaved similarly to glutethimide and to phenobarbitone, which do not reduce sensitivity to pain. It slightly potentiated the analgesic activities of morphine and pethidine in doses of 400 mg./kg. in mice.

Hypothermic and Antipyretic Activities.—Thalidomide did not reduce the basal temperature of rabbits in oral doses of 200 mg./kg. It showed a slight antipyretic activity, lowering and shortening the pyretic response to inactivated *E. coli* bacteria.

Cardiovascular, Respiratory and Autonomic Nervous System

In the anaesthetized cat administration of 125 mg./kg. of thalidomide intraperitoneally or direct into the duodenum did not affect the heart rate, blood pressure or respiration. The blood pressure responses to acetylcholine, adrenaline and histamine were unaltered, and the responses of the nictitating membrane to adrenaline and to stimulation of the preganglionic cervical sympathetic nerve remained the same. The drug therefore had no significant effects on the heart, respiration or autonomic nervous system, nor did it show any antihistamine activity.

The absence of cardiotoxicity was confirmed on the rabbit isolated heart where perfusion with Ringer-Locke solution containing 10 mg./l. of thalidomide produced no change in the amplitude and rate of the contractions. The coronary flow was also unaffected.

Gastro-intestinal Tract

In vitro thalidomide had a mild and short-lived spasmolytic action, a dose of 0.5 mg. in a 15 ml. bath reducing the spasmodic contractions of the guinea-pig isolated ileum to acetylcholine and histamine.

In vivo an oral dose of 500 mg./kg. of thalidomide given 30 min. before a charcoal meal did not significantly reduce the rate of transport through the stomach and intestines. The rate of travel was only reduced by 15%.

Urinary System

Thalidomide did not affect urinary output, the rate of excretion in water-loaded rats being the same after thalidomide as in the controls.

Interaction of Thalidomide with Other Drugs

Central Nervous System Depressants.—(1) Barbiturates. Thalidomide, in common with

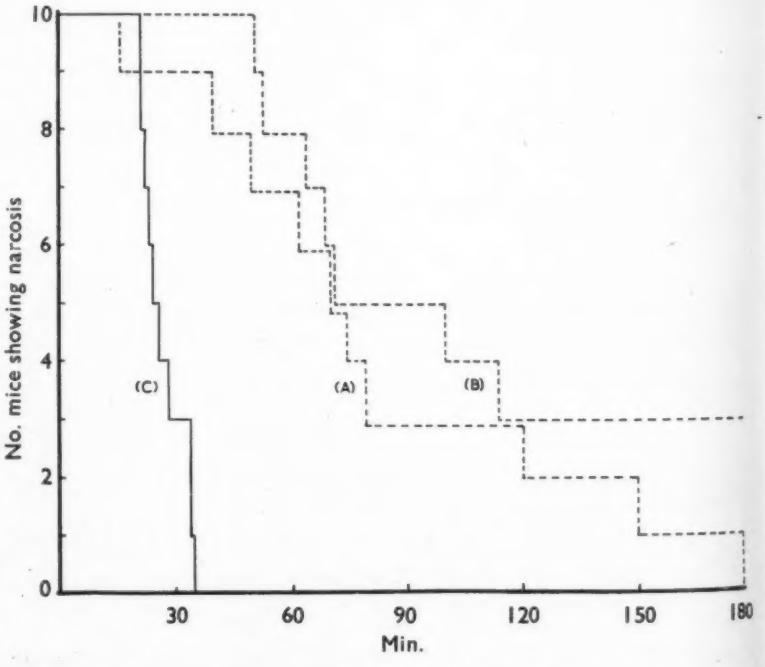

FIG. 3.—The effect of thalidomide (– – – (A) 400 mg./kg.; – – – (B) 1,600 mg./kg.) on hexobarbitone sodium (—— (C) 125 mg./kg.) narcosis in mice.

tranquillizing drugs, potentiated the narcotic action of barbiturates. Fig. 3 illustrates the results from a typical experiment with hexobarbitone showing that the anaesthetic action was considerably prolonged. The mean duration of narcosis in groups of the mice injected with 125 mg./kg. of hexobarbitone intraperitoneally 30 min. after oral doses of 400 and 1,600 mg./kg. of thalidomide were respectively 85 and 98 min. compared with 27 min. for the controls

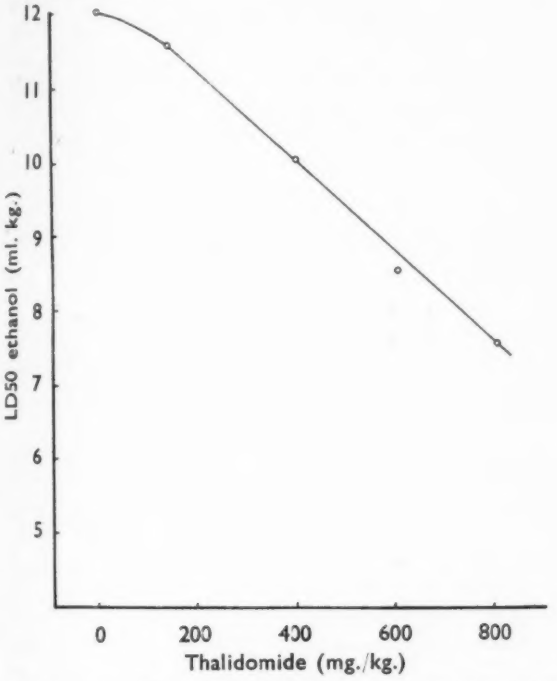

FIG. 4.—The effect of thalidomide on the acute toxicity of ethanol in mice.

TABLE III

THE EFFECT OF THALIDOMIDE ON THE DURATION OF CATATONIA IN MICE GIVEN CHLORPROMAZINE

There were ten mice in each group.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Chlorpromazine (mg./kg.)	Percentage of Mice Showing Catatonia after :	
			24 hr.	48 hr.
1	0	40	50	0
2	100	40	40	0
3	200	40	60	20
4	400	40	90	30
5	800	40	100	90

(2) Alcohol. Thalidomide increased the oral toxicity of ethyl alcohol in mice. The increase in toxicity was linearly related to the dose of thalidomide (Fig. 4).

(3) Reserpine and chlorpromazine. A single oral dose of thalidomide increased the duration of catatonia produced in mice by chlorpromazine or reserpine (Tables III and IV). Similarly thalidomide increased the potentiating action of chlorpromazine and reserpine on hexobarbitone

TABLE IV

THE EFFECT OF THALIDOMIDE ON THE DURATION OF CATATONIA IN MICE GIVEN RESERPINE

There were ten mice in each group.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Reserpine (mg./kg.)	Percentage of Mice Showing Catatonia after 24 hr.
1	Controls	2	0
2	100	2	20
3	200	2	50
4	400	2	60
5	800	2	70

TABLE V

THE EFFECT OF THALIDOMIDE ON THE POTENTIATING ACTION OF CHLORPROMAZINE ON HEXOBARBITAL NARCOSIS IN MICE

There were ten mice in each group. Thalidomide was given 2 hr. before, and chlorpromazine 1 hr. before, hexobarbital. The estimated standard error of a single mean duration of narcosis is ±13%.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Chlorpromazine (mg./kg.)	Intraperitoneal Dose of Hexobarbital (mg./kg.)	Geometric Mean Duration of Narcosis (min.)
1	0	0	125	21·0
2	0	1·5	125	37·9
3	100	1·5	125	36·7
4	200	1·5	125	52·6
5	400	1·5	125	49·4
6	800	1·5	125	70·8
7	100	0	125	30·0
8	200	0	125	30·5
9	400	0	125	33·0
10	800	0	125	44·7

TABLE VI

THE EFFECT OF THALIDOMIDE ON THE POTENTIATING ACTION OF RESERPINE ON HEXOBARBITAL NARCOSIS IN MICE

There were ten mice in each group. Reserpine and thalidomide were given 2 hr. before hexobarbital. The estimated standard error of a single mean duration of narcosis is ±15%.

Group No.	Oral Dose of Thalidomide (mg./kg.)	Intraperitoneal Dose of Reserpine (mg./kg.)	Intraperitoneal Dose of Hexobarbital (mg./kg.)	Geometric Mean Duration of Narcosis (min.)
1	0	0	125	19·4
2	0	0·4	125	31·1
3	100	0·4	125	41·5
4	200	0·4	125	42·2
5	400	0·4	125	47·3
6	800	0·4	125	51·6
7	100	0	125	26·7
8	200	0	125	32·2
9	400	0	125	24·9
10	800	0	125	31·2

narcosis in mice using the test described by Kopmann and Hughes (1958). (Tables V and VI.)

Central Nervous System Stimulants.—(1) Methylamphetamine. This stimulant drug rapidly counteracted the depressant action of thalidomide in mice. They quickly resumed normal motility and with increasing doses of methylamphetamine they became hyperactive and convulsed. The motility of mice made hyperactive with methylamphetamine (5 mg./kg., subcutaneously) was not depressed by an oral dose of thalidomide (500 mg.). The acute toxicity of methylamphetamine was also not reduced by the previous administration of thalidomide.

(2) Methylphenidate ("Ritalin"). The interaction of methylphenidate with thalidomide was very similar to that of methylamphetamine. Very high doses of thalidomide (1 g./kg.) slightly reduced hypermotility of mice given methylphenidate.

DISCUSSION

Thalidomide has been shown experimentally to be a sedative hypnotic drug acting differently from the barbiturates. It does not cause incoordination, respiratory depression or narcosis, and it is virtually non-toxic, possibly due to a limited absorption. The clinical value of this drug has been reported by Jung (1956), Stark (1956) and by Burley, Dennison, and Harrison (1959). Its safety has been confirmed in a number of cases of accidental and suicidal overdoses (de Souza, 1959; Burley, personal communication).

The author thanks Dr. L. Pirbos for histological examinations and Mrs. S. M. Holland, Miss A. Kennedy and Mr. B. Benson for valuable assistance.

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THE ACTION OF SYMPATHETIC BLOCKING AGENTS ON ISOLATED AND INNERVATED ATRIA AND VESSELS

BY

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(RECEIVED JULY 31, 1959)

A preparation is described of isolated rabbit atria with both vagus and sympathetic nerves. The action on it of bretylium and of choline 2,6-xylyl ether bromide (TM10) was studied. A concentration of breylium sufficient to abolish the response to sympathetic stimulation also depressed the response to vagal stimulation. The effect was reversible, though more easily with choline xylyl ether. Both drugs abolished the accelerating action of acetylcholine in the presence of atropine, but they augmented the action of tyramine, and did not reduce that of amphetamine. In the vessels of the perfused rabbit ear they abolished the constrictor effect of nervous stimulation and of acetylcholine, but increased that of tyramine.

The substance choline 2,6-xylyl ether (TM10; hereafter called choline xylyl ether) was synthesized by Hey, and Willey discovered that it blocked the response to stimulation of postganglionic adrenergic fibres without affecting the actions of added adrenaline or noradrenaline (Hey and Willey, 1954). This action was studied in greater detail by Exley (1957). Recently bretylium, *N-o*-bromobenzyl-*N*-ethyl-*NN*-dimethylammonium *p*-toluene sulphonate, has been introduced by Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim (1959) as a substance having a similar action which can be used clinically. The action of these two substances on the isolated atria of the rabbit heart has been examined using in some experiments the sympathetically innervated preparation which has been recently described (Huković, 1959), and, in some, a doubly innervated preparation so that the vagal fibres or the sympathetic fibres could be stimulated at will. The substances have also been examined for their action on the perfused vessels of the rabbit ear.

METHODS

In all experiments on the atria the solution used was: NaCl 9.0 g., KCl 0.42 g., $CaCl_2$ 0.24 g., dextrose 2.0 g. and $NaHCO_3$ 0.5 g. in 1 litre. The temperature of the bath was 30°. The bath was bubbled with O_2. Rabbit's and guinea-pig's atria were set up in the isolated organ bath, as described by Burn (1952).

Rabbit atria with the vagus nerve (McEwen, 1956) and with the sympathetic nerve were prepared as follows. Rabbits were stunned and killed, and quickly tied out on a table. The skin was removed from the thorax and neck. The vagus nerve and the sympathetic chain of the right side were identified and tied. The trachea and the oesophagus were divided as they entered the thorax. The ventral wall of the chest was removed without disturbing the thymus and neighbouring tissues. The vertebral column was divided with strong scissors between the cervical and thoracic vertebrae. The main vessels were divided in front of the 3rd thoracic vertebra, and the vertebral column was again divided between the 2nd and 3rd thoracic vertebrae. All skeletal muscles were removed and the preparation was immersed in oxygenated solution at 30°. The preparation was left in this solution at least 5 min. The preparation was then cleaned again and fixed on the short part of an L-shaped rod, and then was again immersed in the solution where it remained. The proximal part of the vagus nerve was found and tied. The cervical sympathetic nerve was tied and freed of connective tissue as far as the stellate ganglion by blunt dissection, freeing the ganglion as well as possible. The vagus nerve was placed in one pair of electrodes approximately in the middle of its length. The second pair of electrodes were placed around the stellate ganglion itself. The electrodes were of a pattern described by Burn and Rand (1959) with a channel in the holder through which a small amount of solution was constantly siphoned from the bath so that the portion of nerve stimulated was continually irrigated by fully oxygenated solution. The other end of the atria was tied to a spring lever. The stimulus applied was 0.5 mA strength, at a frequency of 20 pulses/sec. Each stimulus was 5 msec. duration, and stimulation was maintained for 15 to 30 sec.

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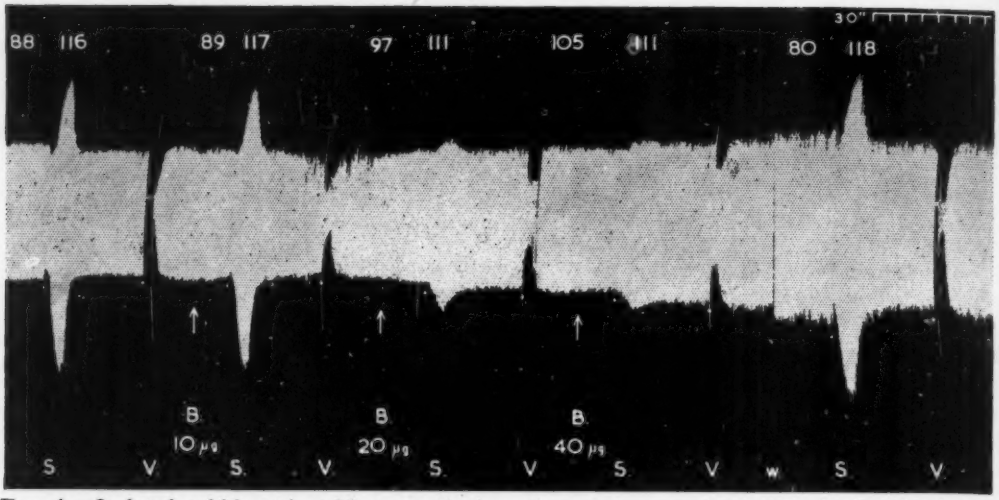

FIG. 1.—Isolated rabbit atria with sympathetic and vagus nerves. S, sympathetic stimulation, showing increase in rate (beats/min.; figures above the tracing) and amplitude. V, vagus stimulation, showing inhibition. B, addition of bretylium, the concentration per ml. being given. Note that in the concentration of 40 μg./ml., bretylium nearly abolished the effect of sympathetic stimulation and in addition reduced the effect of vagal stimulation. W, washing out of bath many times with recovery of effect of sympathetic and vagal stimulation.

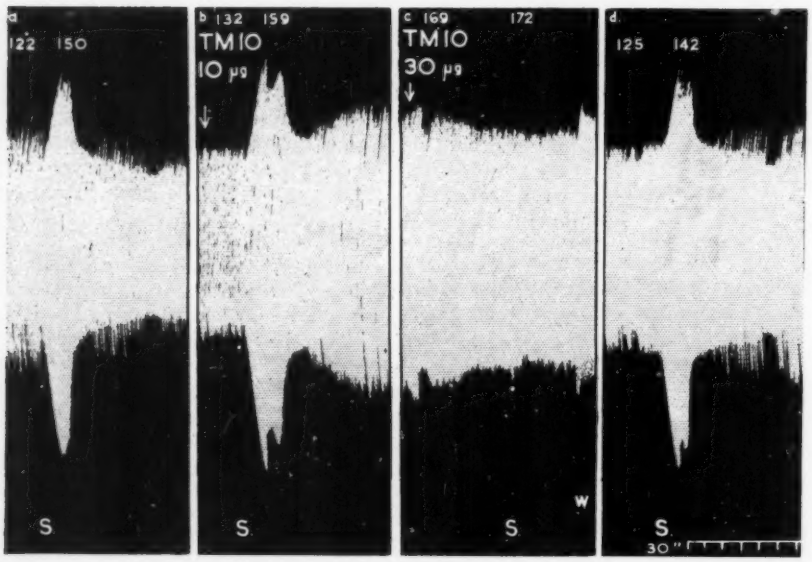

FIG. 2.—Isolated rabbit atria, showing block of effect of sympathetic stimulation (S) by choline xylyl ether (TM10). After 3 min. in bath 10 μg./ml. had no effect (second panel). A further 10 μg./ml. was added 5 min. later, making 20 μg./ml. in all; 3 min. after this second addition, the effect of sympathetic stimulation was diminished but not abolished (not shown). After 5 min. a third addition of 10 μg./ml. was made, making 30 μg./ml. in all. The effect of nerve stimulation was now abolished (third panel). After four washings (W) over 20 min., the effect of nerve stimulation was restored (last panel).

Rabbit ears were perfused with Locke solution of the composition described above with half the amount of dextrose. The arrangement was that described by Burn (1952) using Stephenson's recorder (1948). Stimulation was applied by a pair of electrodes to the great auricular nerve at the base of the pinna.

RESULTS

Bretylium in Doubly Innervated Preparation.—The effect of bretylium in isolated rabbit atria innervated by the vagi and by the sympathetic nerves is shown in Fig. 1. Stimulation of the sympathetic nerves increased the amplitude and also increased the rate from 88 to 116 beats/min. Stimulation of the vagus caused atrial arrest. Bretylium added in a concentration of 10 μg./ml. had no effect on sympathetic stimulation, but diminished the effect of vagal stimulation; in a concentration of 20 μg./ml., however, it diminished the effect of sympathetic stimulation without much further effect on vagal stimulation. Finally

bretylium in a concentration of 40 μg./ml. nearly abolished the effect of sympathetic stimulation; whereas the initial rise in rate was 28 beats/min., it was reduced to 6 beats/min. However, the effect of vagal stimulation was also smaller. After removing the bretylium from the bath, the initial effects both of sympathetic and of vagal stimulation were restored after many washings during 1 hr.

Choline Xylyl Ether on Sympathetic Stimulation. — The effect of choline xylyl ether on sympathetic stimulation is shown in Fig. 2. At first stimulation increased the rate by 28 beats/min. In the presence of 10 μg./ml. stimulation was unaffected, but a higher concentration, 30 μg./ml., blocked the effect of stimulation. After washing out the drug, the initial effect of stimulation was restored much more readily than when bretylium was used.

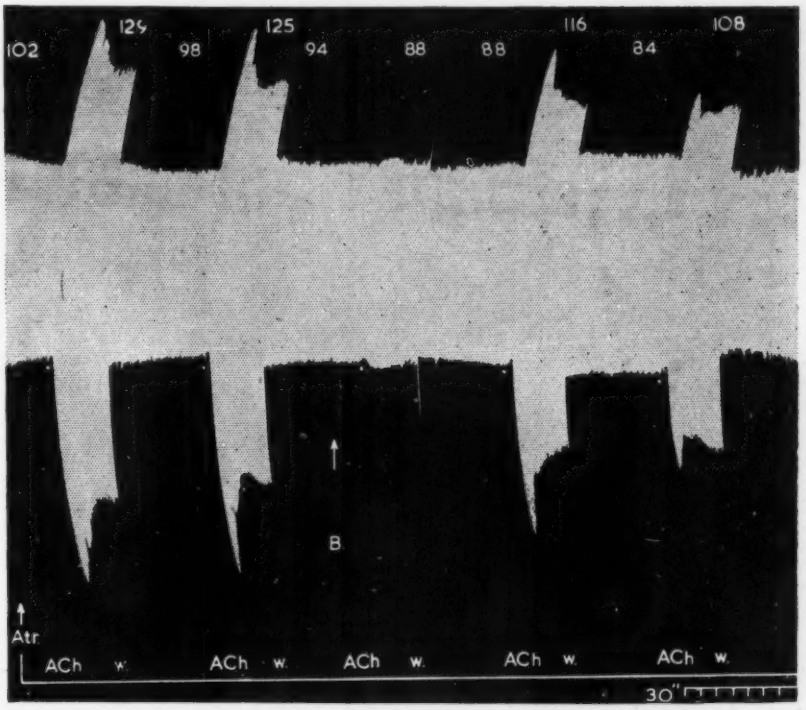

FIG. 3.—The effect of bretylium (**B**) in preventing the increase in rate and amplitude due to acetylcholine (**ACh**) in the presence of atropine. Atropine (**Atr**) 5 μg./ml., acetylcholine 200 μg./ml., bretylium 20 μg./ml. **W**, washing out.

The Response to Acetylcholine.—In the presence of atropine, acetylcholine causes stimulation of the isolated rabbit atria as shown in Fig. 3. The concentration of atropine was 5 μg./ml., and the concentration of acetylcholine was 200 μg./ml. This caused the rate to increase by 27 beats/min. in two successive trials. Bretylium (20 μg./ml.) was then added and in its presence acetylcholine was without effect. On washing out the bretylium the effect of acetylcholine was quickly restored. The action of choline xylyl ether was similar to that of bretylium; it also blocked the action of acetylcholine.

The Response to Tyramine and Amphetamine.—Concentrations of choline xylyl ether and of bretylium which blocked the accelerator action of acetylcholine had no blocking action on the accelerating action of tyramine. This is illustrated in Fig. 4. A concentration of tyramine, 2 μg./ml., applied twice caused a rise in rate of 16 and of 14 beats/min.; in the presence of bretylium, 20 μg./ml., tyramine, 2 μg./ml., caused a rise in rate of 23 beats/min.

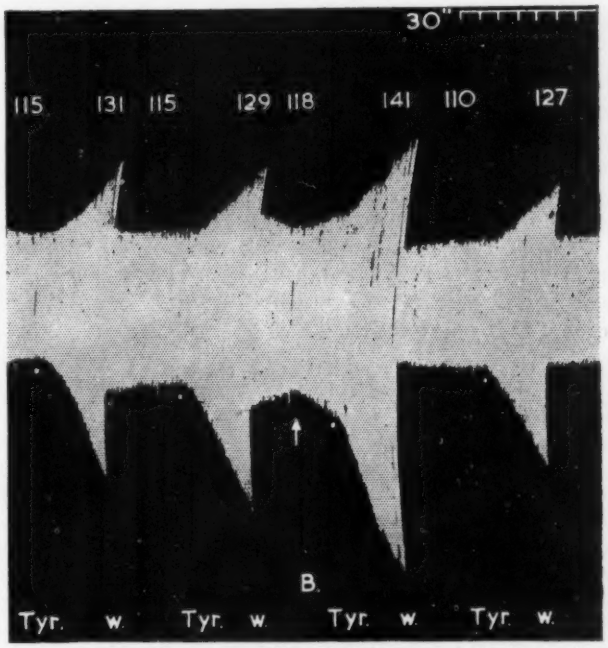

FIG. 4.—The effect of bretylium (**B**), 20 μg./ml., in increasing the effect of tyramine (**Tyr**), 2 μg./ml., on the rate and amplitude of the atria. **W**, washing out.

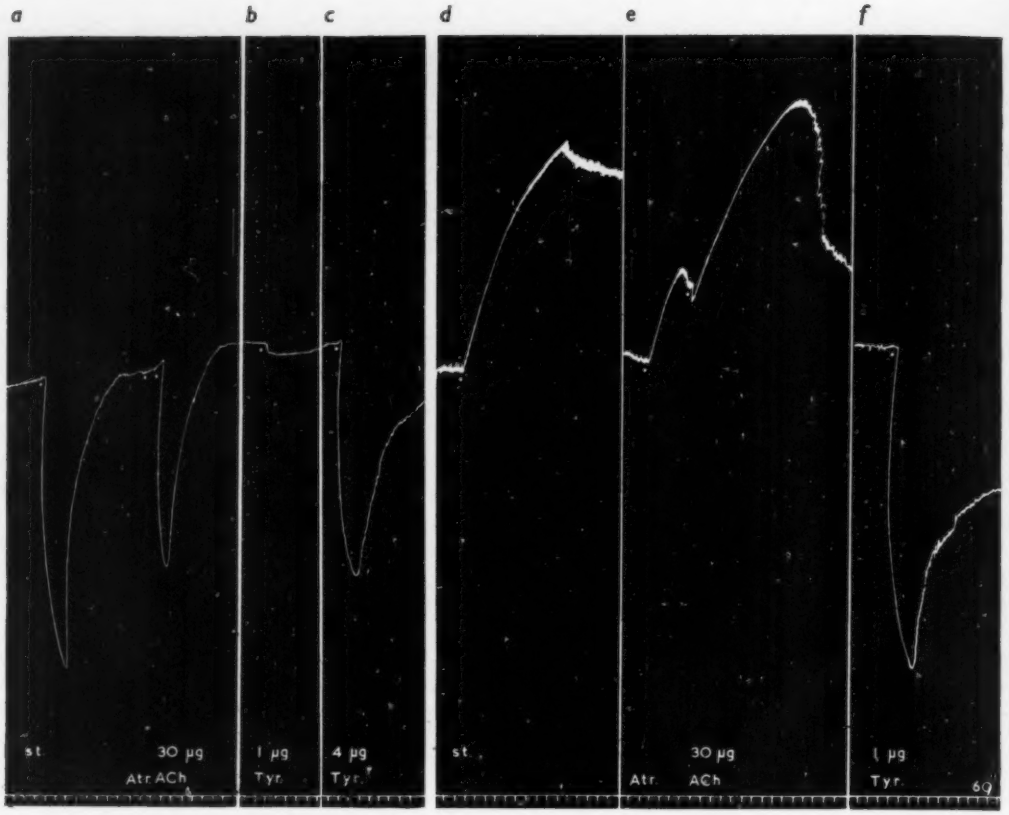

FIG. 5.—Record of outflow from veins of rabbit ear perfused with Locke solution at room temperature. **a,** Fall in outflow due to stimulation of great auricular nerve at the base of the ear (**st**); also fall in outflow due to injection of 30 µg. acetylcholine (**ACh**) after 8 µg. of atropine (**Atr**) into the solution entering the artery. **b,** No change in outflow after the injection of 1 µg. tyramine. **c,** Fall in outflow after injection of 4 µg. tyramine. The ear was then perfused overnight with Locke solution containing 1 µg./ml. bretylium. **d,** Rise in outflow following stimulation of the nerve. **e,** Rise in outflow following injection of 30 µg. acetylcholine after 8 µg. atropine. **f,** Fall in outflow after injection of 1 µg. tyramine.

After washing out the bretylium a further application of tyramine caused a rise in rate of 17 beats/min. Thus bretylium increased the action of tyramine.

The action of amphetamine in increasing the amplitude and rate of the atria was likewise not blocked by bretylium. Since the action of amphetamine is prolonged, a comparison was made between the action of amphetamine in one atrial preparation with its action in the presence of bretylium in another preparation. No difference was observed. In the presence of bretylium, 20 µg./ml., amphetamine, 5 µg./ml., increased the rate from 86 to 132 beats/min.

Vessels of Rabbit Ear.—Similar observations were made on the vessels of the rabbit ear. Bretylium and choline xylyl ether when added to the perfusion fluid blocked the constrictor effect of stimulating the great auricular nerve and also that of acetylcholine in the presence of atropine. However, they increased the effect of tyramine. Fig. 5 illustrates these actions of bretylium when it was present in the perfusion fluid in a concentration of 1 µg./ml. and had been perfused throughout the night. The dilator effect of stimulation in the presence of bretylium may have been due to stimulation of sensory fibres in the mixed nerve. The dilator action of acetylcholine in the presence of bretylium may have been due to the administration of insufficient atropine.

DISCUSSION

The use of an isolated atrial preparation with both vagal and sympathetic nerves intact made

it possible to compare the action of bretylium on the two innervations. In a concentration which almost completely blocked the effect of stimulating sympathetic fibres there was a considerable diminution in the effect of vagal stimulation also. Hence the selective action on the sympathetic terminations was only partial.

In a previous paper (Huković, 1959) it has been shown that when atrial preparations were made from rabbits treated with reserpine, stimulation of the sympathetic fibres often caused inhibition, and that this inhibitory effect was increased by eserine and abolished by atropine. This showed that cholinergic fibres were present. As Burn and Rand (1959) have suggested, such cholinergic fibres would normally have an adrenergic effect, since the acetylcholine they liberate would in turn liberate noradrenaline from the store in the neighbourhood of the nerve endings. These cholinergic fibres are indeed only recognized as cholinergic when treatment with reserpine has dispersed the store, and the liberated acetylcholine then exerts its own effect. Choline xylyl ether and bretylium inhibit the accelerating action of acetylcholine seen in the presence of atropine. This action has been shown to be exerted by release of noradrenaline from the store, since it is absent in preparations from rabbits treated with reserpine. Choline xylyl ether and bretylium do not appear to affect the store of noradrenaline itself, since they do not reduce the action of tyramine, and indeed they increase it. It is suggested, therefore, that they inhibit the accelerating action of acetylcholine mentioned above by preventing the liberation of noradrenaline from the store. They would also be expected to inhibit the noradrenaline-liberating action of cholinergic nerve fibres.

This work has been done during the tenure of a Fellowship from the British Council. I am indebted to Miss Roneen Hobbs for the experiments on the perfused rabbit's ear. This work was suggested and supervised by Professor J. H. Burn, to whom I am very grateful.

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ACTION OF TWO ETHYL THIOL ESTERS AGAINST EXPERIMENTAL TUBERCULOSIS IN THE GUINEA-PIG

BY

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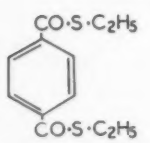
(RECEIVED OCTOBER 2, 1959)

Two thiol esters, ethyl dithiolterephthalate (Compound 13,130) and ditophal (ethyl dithiolisophthalate; Etisul), have been tested for their antituberculosis activity in guinea-pigs. Compound 13,130 was active at a dose of 100 mg./kg. twice daily by mouth in animals infected subcutaneously, but was inactive against an intracerebral infection at 50 mg./kg. Ditophal was active in the subcutaneously infected animals at 200 mg./kg. orally or 50 mg./kg. subcutaneously. Higher doses subcutaneously were irritant.

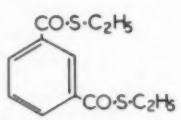
We have already described the antituberculosis effect of some thiol esters against experimental infections in mice (Davies, Driver, Hoggarth, Martin, Paige, Rose, and Wilson, 1956). Ditophal (ethyl dithiolisophthalate; Etisul) exerted an antituberculosis effect in mice comparable to that of isoniazid and streptomycin. It was effective when given in single doses, and against isoniazid-resistant strains. Ethyl dithiolterephthalate (Compound 13,130) is less effective than ditophal but nevertheless is a potent drug. In the present experiments we describe the action of the compounds on experimental tuberculosis in the guinea-pig, an animal in which the course of the disease is modified by the development of hypersensitivity.

METHODS

Two thiol esters have been used:



Compound 13,130



Ditophal

Compound 13,130 is an odourless, tasteless, crystalline solid; ditophal is a bland, odourless oil.

Subcutaneous Infection

Expt. 1 (Compound 13,130 Given Orally).—20 guinea-pigs, 260 to 404 g. in weight, were divided into five groups of four. The animals were infected subcutaneously over the sternum with 0.1 mg. wet weight of *Mycobacterium tuberculosis*, human strain, type C, suspended in 0.1 ml. of distilled water. The suspension was made by ball-milling bacilli harvested from a 14-day culture on Lowenstein's medium. Treatment was arranged as follows:

1. Two doses of 100 mg./kg. Compound 13,130 orally by stomach tube daily from the day of infection.
2. Two doses of 100 mg./kg. Compound 13,130 orally by stomach tube daily, starting from the 14th day after infection.
3. One dose of 20 mg./kg. streptomycin per animal subcutaneously daily, starting from the 14th day after infection.
4. Untreated controls.
5. Untreated controls.

Treatment ceased on the 90th day after infection. In this and all subsequent experiments the animals were not treated on Saturdays and Sundays.

Records were kept of weights, tuberculin reactions, appearance and ulceration of abscesses at the site of injection, enlargement of axillary lymph nodes and survival time.

All animals were examined *post mortem* and the degree of tuberculosis in the lungs, tracheo-bronchial lymph nodes, liver and spleen was estimated, each organ being given a maximum score of 5.

Two control animals were killed and examined on the 14th day in order to assess the degree of tuberculosis at the start of dosing in groups 2 and 3.

The experiment was terminated 120 days after infection.

Expt. 2 (Ditophal Given Orally).—40 guinea-pigs, 434 to 648 g. in weight, were divided into ten groups of four. The experiment was conducted as in Expt. 1, with the following dose schedules:

1. Two doses of ditophal, 200 mg./kg. orally daily from the day of infection.

2. Two doses of ditophal, 200 mg./kg. orally daily from 14 days after infection.
3. Two doses of ditophal, 100 mg./kg. orally daily from the day of infection.
4. Two doses of ditophal, 100 mg/kg. orally daily from 14 days after infection.
5. One dose of streptomycin, 40 mg./kg. subcutaneously daily from the day of infection.
6. One dose of streptomycin, 40 mg./kg. subcutaneously daily from 14 days after infection.
7. Two doses of isoniazid, 5 mg./kg. orally daily from the day of infection.
8. Two doses of isoniazid, 5 mg./kg. orally daily from 14 days after infection.
9. Untreated controls.
10. Untreated controls.

Expt. 3 (Ditophal Given Subcutaneously).—48 guinea-pigs, 440 to 630 g. in weight, were divided into six groups of eight and the experiment was carried out as in Expt. 1 with the treatment as follows:

1. Ditophal, 50 mg./kg. subcutaneously, once daily.
2. Ditophal, 50 mg./kg. subcutaneously, once weekly.
3. Ditophal, 10 mg./kg. subcutaneously, once daily.
4. Ditophal, 10 mg./kg. subcutaneously, once weekly.
5. Streptomycin, 40 mg./kg. subcutaneously, once daily.
6. Untreated controls.

Treatment ceased on the 120th day after infection.

Intracerebral Infection

Expt. 4 (Compound 13,130 Given Orally).—32 guinea-pigs, 300 to 400 g. in weight, were divided into four groups of eight and infected, under ether anaesthesia, by the intracerebral injection of 0.01 mg. wet weight of *M. tuberculosis* " Human C," suspended in 0.1 ml. of distilled water.

Treatment was started on the day after infection and was continued until death or for 5 weeks. The brains were removed *post mortem* and stained smears examined for the presence of *M. tuberculosis.* All animals were weighed once weekly.

TABLE I

THE EFFECT OF COMPOUND 13,130 AND STREPTOMYCIN ON GUINEA-PIGS INFECTED SUBCUTANEOUSLY

S=animal alive 120 days after infection. K14=animal killed at 14 days for assessment of degree of tuberculosis.

Group	Animal No.	Treatment	Degree of Tuberculosis (Maximum 5)					Survival Time in Days
			Lung	Liver	Spleen	Bronchial Lymph Nodes	Total	
1	1	Compound 13,130, two doses of 100 mg./kg. orally daily from day of infection	3	2	4	5	14	S
	2		4	3	5	5	17	S
	3		2	2	4	5	13	S
	4		2	2	5	5	14	S
	Mean		2·75	2·25	4·5	5·0	14·5	120
2	1	Compound 13,130, two doses of 100 mg./kg. orally daily from 14 days after infection	4	3	5	5	17	S
	2		1	1	2	2	6	S
	3		3	5	5	5	18	112
	4		1	1	5	5	12	S
	Mean		2·25	2·5	4·25	4·25	13·25	118
3	1	Streptomycin 20 mg./kg. subcutaneously once daily from 14 days after infection	0	0	0	0	0	S
	2		0	3	5	5	13	103
	3		1	1	5	5	12	S
	4		4	5	5	5	19	S
	Mean		1·25	2·25	3·75	3·75	11·0	116
4 and 5	1	Controls	0	0	0	5	5	K14
	2		0	0	0	5	5	K14
	3		5	5	5	5	20	76
	4		3	3	5	5	16	81
	5		4	3	3	5	15	70
	6		5	5	5	5	20	88
	7		4	2	3	5	14	S
	8		3	5	5	3	16	51
	Mean		4·0	3·84	4·33	4·66	16·8	81·0

TABLE II

THE EFFECT OF DITOPHAL GIVEN ORALLY, STREPTOMYCIN AND ISONIAZID ON GUINEA-PIGS INFECTED SUBCUTANEOUSLY

NS = death from some non-specific cause. S = alive 120 days after infection

Group	Animal No.	Treatment	Degree of Tuberculosis (Maximum 5)					Survival Time in Days
			Lung	Liver	Spleen	Bronchial Lymph Nodes	Total	
1	1	Ditophal, two doses 100 mg./kg. orally daily from day of infection	2	1	2	5	10	65
	2		1	0	2	0	3	18 NS
	3		3	1	3	5	12	S
	4		2	2	3	5	12	59
	Mean		2·3	1·3	2·7	5·0	11·3	81·3
2	1	Ditophal two doses of 100 mg./kg. orally daily from 14 days after infection	0	0	2	0	2	21 NS
	2		1	0	2	0	3	49 NS
	3		2	1	3	5	11	S
	4		0	0	2	5	7	74
	Mean		1·0	0·5	2·5	5·0	9·0	97·0
3	1	Ditophal two doses of 200 mg./kg. orally daily from day of infection	1	0	2	3	6	S
	2		1	0	1	5	7	S
	3		0	0	2	0	2	41 NS
	4		2	0	3	5	10	59
	Mean		1·3	0	2·0	4·3	7·7	99·7
4	1	Ditophal two doses of 200 mg./kg. orally daily from 14 days after infection	4	2	3	5	14	S
	2		2	2	1	5	10	S
	3		1	0	0	0	1	44 NS
	4		1	0	0	2	3	S
	Mean		2·3	1·3	1·3	4·0	9·0	120
5	1	Streptomycin 40 mg./kg. subcutaneously once daily from day of infection	0	0	0	2	2	S
	2		1	0	2	5	8	S
	3		1	0	0	1	2	S
	4		0	0	0	1	1	S
	Mean		0·5	0	0·5	2·3	3·3	120
6	1	Streptomycin 40 mg./kg. subcutaneously once daily from 14 days after infection	1	0	0	1	2	S
	2		0	0	0	0	0	S
	3		0	0	2	5	7	58 NS
	4		0	0	2	0	2	54 NS
	Mean		0·5	0	0	0·5	1·0	120
7	1	Isoniazid, two doses of 5 mg./kg. orally daily from day of infection	0	0	0	0	0	S
	2		0	0	0	0	0	S
	3		0	0	0	0	0	29 NS
	4		0	0	0	0	0	29 NS
	Mean		0	0	0	0	0	120
8	1	Isoniazid, two doses of 5 mg./kg. orally daily from 14 days after infection	0	0	0	0	0	S
	2		0	0	0	0	0	S
	3		0	0	0	0	0	S
	4		0	0	0	0	0	S
	Mean		0	0	0	0	0	120
9 and 10	1	Controls	3	5	5	5	18	87
	2		2	3	3	5	13	54
	3		5	5	5	5	20	92
	4		3	5	5	5	18	37
	5		5	5	5	5	20	97
	6		2	5	5	5	17	56
	7		4	5	5	5	19	54
	8		5	5	3	5	18	S
	Mean		3·6	4·8	4·5	5·0	17·9	74·6

TABLE III

THE EFFECT OF DITOPHAL GIVEN SUBCUTANEOUSLY AND STREPTOMYCIN ON GUINEA-PIGS INFECTED SUBCUTANEOUSLY

NS = death from some non-specific cause. S = alive 120 days after infection.

Group	Animal No.	Treatment	Degree of Tuberculosis (Maximum 5)					Survival Time in Days
			Lung	Liver	Spleen	Bronchial Lymph Nodes	Total	
1	1	Ditophal 50 mg./kg. subcutaneously once daily	1	2	5	4	12	S
	2		2	5	5	5	17	111
	3		3	4	5	5	17	S
	4		2	3	5	5	15	S
	5		1	3	4	5	13	S
	6		2	2	4	4	12	S
	7		1	5	2	4	12	S
	Mean		1·7	3·4	4·3	4·6	14·0	118
2	1	Ditophal 50 mg./kg. subcutaneously once weekly	4	4	4	5	17	53
	2		5	5	5	5	20	93
	3		5	2	2	3	12	109
	4		2	4	3	4	13	70
	5		5	5	5	5	20	S
	6		1	5	5	5	16	52
	7		5	5	3	4	17	102
	8		4	4	3	4	15	109
	Mean		3·9	4·25	3·8	4·4	16·2	88·6
3	1	Ditophal 10 mg./kg. subcutaneously once daily	5	4	5	4	18	S
	2		5	4	3	5	17	S
	3		4	5	5	5	19	77
	4		5	4	3	5	17	88
	5		3	4	3	4	14	65
	6		4	2	2	4	12	S
	7		1	2	2	2	7	S
	8		4	5	5	4	18	92
	Mean		3·9	3·75	3·5	4·1	15·25	100
4	1	Ditophal 10 mg./kg. subcutaneously once weekly	5	1	1	5	12	98
	2		5	3	3	3	14	105
	3		5	5	4	5	19	68
	4		1	0	0	5	6	94
	5		4	2	2	3	11	S
	6		5	5	5	5	20	80
	7		4	2	3	4	13	S
	8		4	2	4	4	14	S
	Mean		4·1	2·5	2·75	4·25	13·6	100
5	1	Streptomycin 40 mg./kg. subcutaneously once daily	0	0	0	0	0	S
	2		0	0	0	0	0	S
	3		0	0	0	0	0	S
	4		0	0	0	0	0	S
	5		0	0	0	0	0	S
	6		0	0	0	0	0	S
	7		0	0	0	0	0	S
	8		0	0	0	0	0	S
	Mean		0	0	0	0	0	120
6	1	Controls	5	5	5	5	20	97
	2		5	5	4	5	19	S
	3		5	5	5	5	20	99
	4		3	5	5	5	18	49
	5		5	5	5	5	20	94
	6		3	3	5	5	16	S
	7		1	1	4	3	9	S
	8		4	5	4	4	17	75
	Mean		3·95	4·25	4·6	4·6	17·4	97

TABLE IV

THE EFFECT OF COMPOUND 13,130 ON THE SURVIVAL TIMES OF GUINEA-PIGS INFECTED INTRACEREBRALLY

S=animal surviving 60 days after infection. NS=death from some non-specific cause.

Group	Treatment	Survival Time in Days of Individual Animals								Mean Survival Time in Days
1	Compound 13,130, two doses of 50 mg./kg. orally daily	13	17	19	19	19	20	21	23	19
2	Compound 13,130, two doses of 50 mg./kg. orally twice daily+streptomycin 40 mg./kg. subcutaneously once daily	20	20	32	S	S	S	S	S	>44
3	Streptomycin 40 mg./kg. subcutaneously once daily	30	46	52	56	S	S	S	S	>49
4	Controls	12	15	NS 17	17	19	21	22	22	18

TABLE V

THE EFFECT OF DITOPHAL DOSED ORALLY ON THE SURVIVAL TIMES OF GUINEA-PIGS INFECTED INTRACEREBRALLY

NS=death from some non-specific cause.

Group	Treatment	Survival Time in Days of Individual Animals								Mean Survival Time in Days
1	Ditophal, two doses of 200 mg./kg. orally daily ..	1 NS	12 NS	13	13	26	27	43	49	28·5
2	,, ,, 100 ,, ,, ..	13	16	19	36	37	42	46	71	35·0
3	Streptomycin, two doses of 40 mg./kg. subcutaneously twice daily	19	22	22	26	40	43	44	56	34·0
4	Isoniazid, two doses of 5 mg./kg. orally daily ..	22 NS	39 NS	48	53	54	59	61	71	57·7
5	Untreated controls	15	15	17	19	19	19	21	23	18·6

The four groups were treated as follows:

1. Compound 13,130, 50 mg./kg. orally twice daily.
2. Compound 13,130, 50 mg./kg. orally twice daily plus 40 mg./kg. streptomycin subcutaneously once daily.
3. Streptomycin, 40 mg./kg. subcutaneously once daily.
4. Untreated controls.

Expt. 5 (Ditophal Dosed Orally).—40 guinea-pigs, 238 to 472 g. in weight, were arranged in groups of eight so that the mean weights of each group at the start of the experiment were roughly equal. They were infected intracerebrally, under ether anaesthesia, with 0.01 ml. mg. wet weight *M. tuberculosis,* suspended in 0.1 ml. of distilled water.

Treatment was started on the day after infection and continued for four weeks. The brains were removed *post mortem* and stained smears examined for the presence of *M. tuberculosis.* All animals were weighed once weekly.

The five groups were treated as follows:

1. Ditophal, 200 mg./kg. orally twice daily
2. Ditophal, 100 mg./kg. orally twice daily.
3. Streptomycin, 40 mg./kg. subcutaneously once daily.
4. Isoniazid, 5 mg./kg. orally twice daily.
5. Untreated controls.

RESULTS

Subcutaneous Infection

Both compounds showed a therapeutic effect against a subcutaneous infection even when given against an established infection, but both were less effective than streptomycin. (See Tables I, II, and III.)

The two thiol esters and streptomycin had no effect on the development of the tuberculin reaction or on the time of appearance and subsequent development of the abscesses at the site of inoculation or on the enlargement of the axillary lymph nodes. This is in contrast to the results obtained with isoniazid, where a small abscess appeared in only one guinea-pig and there was no enlargement of the axillary lymph nodes.

Intracerebral Infection

Compound 13,130 had no effect on an intracerebral infection of *M. tuberculosis* in guinea-pigs when dosed twice daily at 50 mg./kg. orally, nor did it potentiate the effect of streptomycin against such an infection (see Table IV).

Ditophal had a definite effect against an intracerebral infection when administered twice daily at 100 mg./kg. and 200 mg./kg. orally, which was comparable with that produced by streptomycin administered subcutaneously at 40 mg./kg. daily, but was inferior to the effect obtained with isoniazid administered twice daily at 5 mg./kg. (see Table V). Ditophal was without therapeutic effect when two weekly doses of 100 mg./kg. were given intracerebrally, or when given once daily subcutaneously at 50 and 10 mg./kg. or once weekly subcutaneously at 100 and 200 mg./kg.

Discussion

The striking antituberculosis effect obtained with ditophal in mice was not achieved in guinea-pigs. This was probably because the maximum dose of ditophal tolerated in guinea-pigs was limited by ulceration of the site of injection with doses greater than 50 mg./kg. The results do, however, indicate that the two thiol esters possess definite activity against tuberculosis in the guinea-pig, and further work with them seems justified.

Etisul is a trade mark of Imperial Chemical Industries.

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THE EFFECTS OF AMBUCETAMIDE ON HUMAN MYOMETRIAL AND OTHER PREPARATIONS, AND ITS ANTAGONISM TO THE MENSTRUAL STIMULANT

BY

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A plain-muscle stimulant, provisionally named the " menstrual stimulant," may be involved in the causation of dysmenorrhoea. Since ambucetamide [α-dibutylamino-α-(*p*-methoxyphenyl)-acetamide] has been claimed to alleviate this condition, its effects on the responses of human myometrial preparations to the menstrual stimulant have been studied. Ambucetamide regularly inhibited these responses, as well as those to vasopressin, and sometimes diminished the spontaneous rhythmical activity of the human myometrium. The rat and guinea-pig uterus, the guinea-pig and cat intestine preparations, showed more complex responses and may therefore be less suitable for testing antispasmodic drugs intended for treatment of dysmenorrhoea.

Ambucetamide [α-dibutylamino-α-(*p*-methoxyphenyl)acetamide; Dibutamide] is an antispasmodic drug which has been found to have some use in alleviating dysmenorrhoea (Carpenter and Janssen, 1955; Hoekstra, Fisher, Cull, Tisch, and Dickison, 1957). The " menstrual stimulant " is a plain-muscle stimulant of endometrial origin, overactivity of which is considered to be a possible cause of dysmenorrhoea (Chambers and Pickles, 1958; Pickles, 1959). It was therefore of interest to see whether ambucetamide antagonized the effect of menstrual stimulant on the human myometrium. If so, it would be of value to see whether the antagonism can be demonstrated on mammalian tissues which are more readily available in the laboratory.

A preliminary account of some of these findings has been reported (Clitheroe and Pickles, 1959).

METHODS

Human myometrial preparations, guinea-pig or rat uterus or guinea-pig duodenum preparations were suspended in a solution containing NaCl 0.82%, KCl 0.052%, $CaCl_2$ 0.008%, $NaHCO_3$ 0.047%, glucose 0.1%, and $MgSO_4$ 0.018%, as described by Pickles (1959).

The preparations of " menstrual stimulant " were prepared by making ether extracts of the lipids of human menstrual fluid. They were either unfractionated or treated as the preparation called "chromatographic fraction 5 " in the method of Chambers and Pickles (1958). The results with the two kinds of extract were qualitatively similar.

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RESULTS

Human Myometrium.—In concentrations of 6 to 120 μg./ml., ambucetamide inhibited the responses of the human myometrium to the menstrual stimulant. In Fig. 1, a preparation, which had previously been giving regular responses to the same doses of menstrual stimulant, ceased to give any response after ambucetamide (25 μg./ml.) had been added to the bath 5 min. previously. This experiment was unusual in that the myometrium had been suspended for several hours and was by then showing little spontaneous rhythmical activity, which began again when the ambucetamide was washed out. A different kind of response,

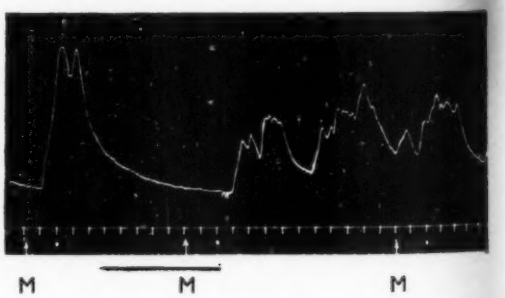

FIG. 1.—Human myometrial preparation *in vitro*. At M, equal doses of ether-soluble menstrual lipid (containing " menstrual stimulant ") were applied for 2 min. White dot indicates washing. Ambucetamide (25 μg./ml.) was applied for duration of horizontal bar. Time, 1 min.

probably bearing more similarity to that *in vivo* but a little more difficult to evaluate pharmacologically, is shown in Fig. 2. This experiment confirms earlier observations (Pickles, 1959) in that a single addition of menstrual stimulant caused the rhythmical contractions of the preparation to become better co-ordinated and more powerful. Such a response might continue almost indefinitely, but in this instance it was inhibited by the addition of ambucetamide.

The usual effect of ambucetamide on the spontaneous activity of a human myometrial preparation is shown in Fig. 3. The tone of the preparation always recovered more slowly than did the amplitude of the spontaneous rhythmical contractions after the ambucetamide was washed out. An inhibitory effect of ambucetamide on the spontaneous activity of the myometrium was clearly seen in about half of the 20 experiments in which it was tested. The antagonism to the menstrual stimulant was seen in each of 13 experiments.

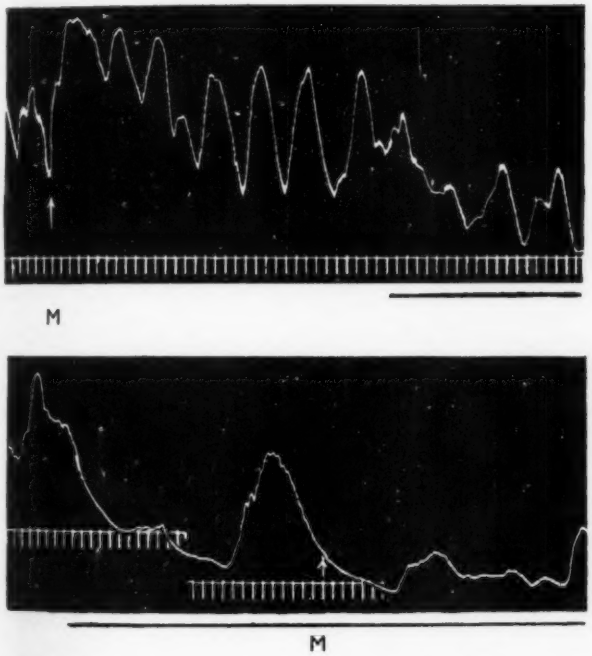

FIG. 2.—Human myometrial preparation *in vitro*. For 45 min. before the section of the record shown in the upper panel, the preparation had shown the poorly co-ordinated contractions as seen in the first 6 min. of the tracing. At M, 3 mg. of ether-soluble menstrual lipid was added to the 8 ml. organ bath; ambucetamide (20 μg./ml.) was added to the bath for the duration of the horizontal bar. The preparation was repeatedly washed during a period of 1.5 hr. between the end of the upper and the beginning of the lower record, but the preparation was not washed during either of the records above. Time, 1 min.

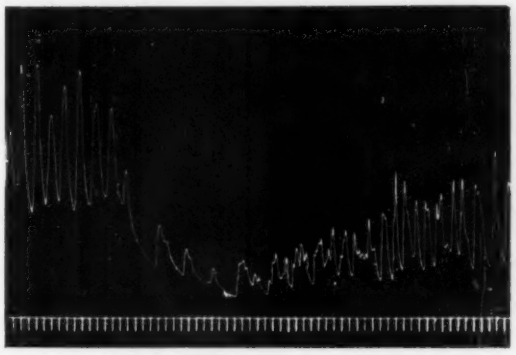

FIG. 3.—Human myometrial preparation *in vitro*. Ambucetamide (25 μg./ml.) was added for the duration of the horizontal bar. Time, 1 min.

In a similar series of 10 experiments, myometrial preparations were stimulated not with the menstrual stimulant but with vasopressin (Pitressin; Parke, Davis & Co.). Again, ambucetamide (12 to 48 μg./ml.) was found to have a long-lasting antagonistic effect.

FIG. 4.—Isolated guinea-pig uterus. The ether-soluble menstrual lipid (0.1 mg.) was added to the 8 ml. organ bath at the black dots for 12 min. at M_1 and for 6 min. at M_2. Ambucetamide (12 μg./ml.) was added for the duration of the horizontal bar. Time, 1 min.

Guinea-pig Uterus. — In 18 experiments on guinea-pig uterus preparations, the stimulating action of menstrual stimulant was antagonized by ambucetamide (12 to 48 μg./ml.) in only three instances; in most of the other experiments, the same concentrations of ambucetamide tended to increase the responses. The guinea-pig uterus normally showed a latency of some minutes in its responses to the menstrual stimulant, but ambucetamide commonly decreased this latency to a few seconds. An example is shown in Fig. 4. In two further experiments, ambucetamide had no definite effect on the responses of the guinea-pig uterus to vasopressin.

Rat Uterus.—Like the guinea-pig uterus, the rat uterus gave variable results. In a series of 9 experiments, ambucetamide (12 to 48 μg./ml.) apparently increased the response to the menstrual stimulant in 4 and decreased it in 5 instances. The effect on the responses to vasopressin was likewise indefinite. Commonly the spontaneous activity increased after the ambucetamide had been washed out.

Guinea-pig Duodenum and Cat Small Intestine. —In both preparations, ambucetamide caused a slow increase in tone while the responses to the menstrual stimulant were diminished.

Discussion

The experiments on the human myometrial preparations are consistent with the finding that ambucetamide is of some value in alleviating dysmenorrhoea. They are also consistent with the view that dysmenorrhoea is due to excessive production of or sensitivity to the menstrual stimulant; but they do not provide positive evidence for this, since ambucetamide also antagonized the effect of vasopressin.

It is clear from the experiments on the isolated uterus of guinea-pigs and rats that these preparations may give responses quite different from those given by the human myometrial preparations. Misleading results might be obtained if guinea-pig or rat uterus preparations are used for testing antispasmodic drugs intended for use in dysmenorrhoea.

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A STUDY OF ANTAGONISTS OF 5-HYDROXYTRYPTAMINE AND CATECHOL AMINES ON THE RAT'S BLOOD PRESSURE

BY

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The effects of 5-hydroxytryptamine on the blood pressure of anaesthetized rats depended on the dose and the initial level of blood pressure. At medium blood pressure levels, 5-hydroxytryptamine gave a depressor response and sometimes a pressor response which was more evident with large doses. The depressor effect was less apparent or even absent at low, and more pronounced at high, blood pressure levels, and the converse applied to the pressor components. Adenosine also gave a depressor and pressor response. Lysergic acid diethylamide, dihydroergotamine, 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (a dichloro analogue of isoprenaline), dibenamine and 1-benzyl-5-methoxy-2-methyltryptamine antagonized 5-hydroxytryptamine and catechol amines. Lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine were more effective against 5-hydroxytryptamine, 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol and dibenamine against catechol amines; dihydroergotamine was equally effective against both groups. These antagonists fell into two groups according to their action against the two types of effects (depressor and pressor) of 5-hydroxytryptamine: lysergic acid diethylamide and 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol acted preferentially against depressor effects; 1-benzyl-5-methoxy-2-methyltryptamine and dibenamine preferentially against pressor; dihydroergotamine was not assignable to either group. Adenosine was affected similarly, but less than 5-hydroxytryptamine.

The effects of 5-hydroxytryptamine on the blood pressure of mammals are complex and vary according to the species (for references see Erspamer (1954) and Page (1958)). In dogs the main effects are pressor while cats and rabbits usually give depressor responses. According to Salmoiraghi, Page, and McCubbin (1956), the responses of rats are intermediate, being mixed pressor and depressor. It was thought useful to study various antagonists (lysergic acid diethylamide, dihydroergotamine, 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (a dichloro analogue of isoprenaline), dibenamine, 1-benzyl-5-methoxy-2-methyltryptamine and atropine) on the mixed responses in rats and to compare the antagonistic action to 5-hydroxytryptamine with that exerted against other drugs (catechol amines, acetylcholine); adenosine was also included as an agonist because it behaved like 5-hydroxytryptamine, and there may be some connexion between 5-hydroxytryptamine and adenosine triphosphate in platelets (Born, Ingram, and Stacey, 1958).

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METHODS

Rats of either sex, between 160 and 200 g. body weight, were used. The anaesthetic used was a mixture of barbitone sodium 10 mg. (as 4% solution) and urethane 75 mg. (as 25% solution), per 100 g. body weight of rat, injected subcutaneously, or half those amounts injected intraperitoneally. In the latter case, if after some time the rat showed signs of coming out of the anaesthetic, a further one fourth of the stated amounts was injected intravenously. The method used was that of Crawford and Outschoorn (1951) and the rats were heparinized. The blood pressure was recorded with a mercury manometer through a cannula in one carotid artery, the opposite carotid artery being tied off. Both vagi and the attendant sympathetic nerve fibres were left intact. Injections were made through a cannula in a femoral vein. In all the experiments the respiration of the animals was spontaneous.

The agonist drugs selected for study and the doses employed were 5-hydroxytryptamine creatinine sulphate, calculated as base, 25 ng. to 20 μg.; adenosine, 50 ng. to 20 μg.; adrenaline hydrochloride as base, 100 to 200 ng.; noradrenaline hydrochloride as base, 50 to 100 ng.; isoprenaline hydrochloride as base, 50 to 100 ng.; acetylcholine chloride as salt,

100 to 500 ng. All doses of the agonist drugs are expressed per rat.

In studies on antagonists the agonist drugs were first given during a control period and the effects recorded. The chosen antagonist was given, at first in small dosage, and the agonists injected again. This process was repeated with increasing doses of the antagonist up to the limits of tolerance of the animal. Such limits were not necessarily the death of the rat, and were usually when the blood pressure had fallen low, compared with that during the control period. At very low blood pressures no conclusions could be drawn because even without antagonists the effects of drugs like acetylcholine were altered. For the experiments with antagonists doses of 0.5 to 2 μg. 5-hydroxytryptamine per rat and 5 to 20 μg. adenosine per rat were chosen. With these doses no tachyphylaxis occurred when the injections were made at intervals of 5 to 10 min.

All the antagonist drugs used, with the exception of atropine were broadly divided into three dose ranges each, as follows: dihydroergotamine methanesulphonate, 5 to 10 μg., 15 to 25 μg., and 35 to 80 μg.; lysergic acid diethylamide, 10 μg., 20 to 40 μg., and 60 to 80 μg.; dibenamine, 50 μg., 100 to 200 μg., and 300 to 400 μg.; 1-benzyl-5-methoxy-2-methyltryptamine hydrochloride, 100 to 200 μg., 300 μg. to 1.2 mg., and 2.0 to 2.8 mg.; 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol hydrochloride, 125 to 500 μg., 750 to 900 μg., and 1.5 to 3.0 mg.; and atropine sulphate, 100 to 200 μg. These doses of antagonists are expressed per 100 g. body weight of rat. In the text the terms small doses, moderate doses and large doses are used to save repetition and refer to the three dose ranges mentioned above.

RESULTS

Effects of 5-Hydroxytryptamine and Adenosine in the Normal Anaesthetized Rat

The effects of 5-hydroxytryptamine and adenosine on the rat blood pressure were complex and depended on the dose and on the initial level of blood pressure.

Dependence on Dosage

With anaesthesia such as to keep the blood pressure at a medium level, doses ranging from the threshold to as high as 20 μg. per rat caused a great variety of responses. An analysis was made of the records at a particular drum speed (10 mm. per min. at the circumference). It would appear that the basic effect of 5-hydroxytryptamine was depressor. Depending on the dose employed, and varying in different rats (Fig. 1), two types of pressor components were superimposed on this depressor response. First there was a short "spike" which occurred early, either at the beginning or at any time during the depressor effect; that is to say the spike interrupted the fall of blood pressure; and second a "dome" which was prolonged and followed the hypotension.

On the basis of this analysis the effects of 5-hydroxytryptamine as related to dose were as follows (Fig. 2, upper record). Doses of 0.5 to 1 μg. per rat usually caused a fall of blood pressure only. Occasionally a spike was also present. The threshold dose was 250 to 500 ng. per rat. The effects of smaller doses (25 ng. per rat) were also observed, but were indistinguishable from injections of saline. Doses of 2 μg. per rat and above usually produced mixed effects. The

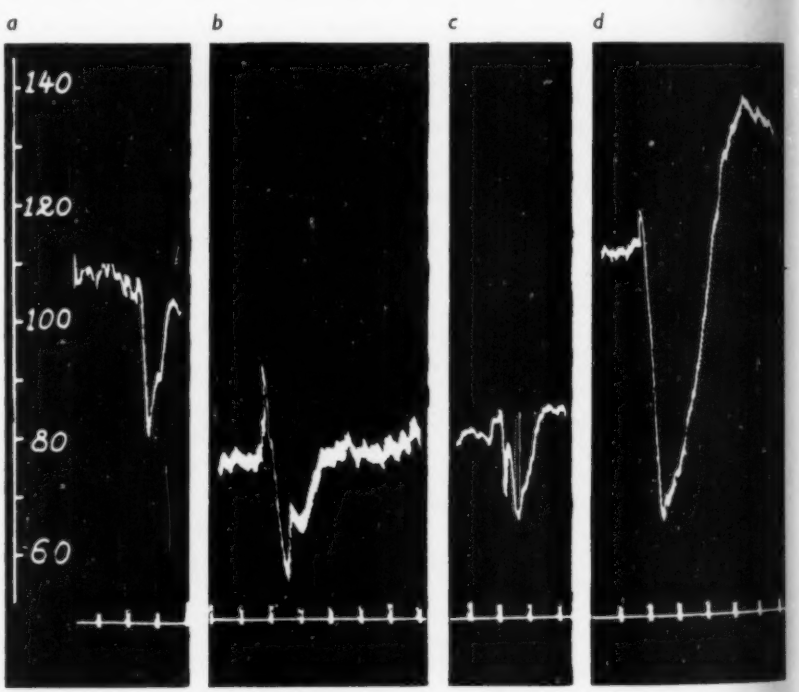

FIG. 1.—Blood pressure records from four different rats, 160, 200, 190 and 185 g., respectively, anaesthetized with urethane-barbitone sodium mixture. The figure illustrates the different responses to 2 μg. 5-hydroxytryptamine, from left to right: (a) depressor effect alone, (b) with superimposed early spike, (c) with late spike, and (d) with spike and dome. Pressure in mm. mercury. Time 0.5 min.

higher the dose the greater the proportion of pressor components. The results of injecting 5 to 10 μg. per rat were taller spikes and larger domes than with 2 μg. per rat. Sometimes the effect of these high doses was to obscure the depressor phase completely. In two rats out of fifty there occurred only pressor effects, whatever the dose of 5-hydroxytryptamine ; in these cases the record consisted of an early spike which was followed by a dome before the blood pressure could return to its original level.

The effects of adenosine were also mixed, and for certain doses they closely resembled those of 5-hydroxytryptamine. However, within a wide dose range they showed a different development of qualitative effects (Fig. 2, lower record). Increasing doses from 50 ng. per rat rarely caused any effect until doses of 2 to 5 μg. per rat were reached. These usually produced a sharp pressor spike. Larger doses (10 to 20 μg. per rat) resulted in a pressor spike being followed by a depressor phase. This was sometimes followed by a pressor dome, as in the case of 5-hydroxytryptamine. Still larger doses (up to 150 μg. per rat) showed a small spike followed by a profound fall. This fall was either prolonged or transient and was followed by a pressor dome. The latter type is illustrated in Fig. 2 (lower record). Thus, increasing the dose of adenosine increased its depressor effects, while increasing the dose of 5-hydroxytryptamine increased its pressor effects.

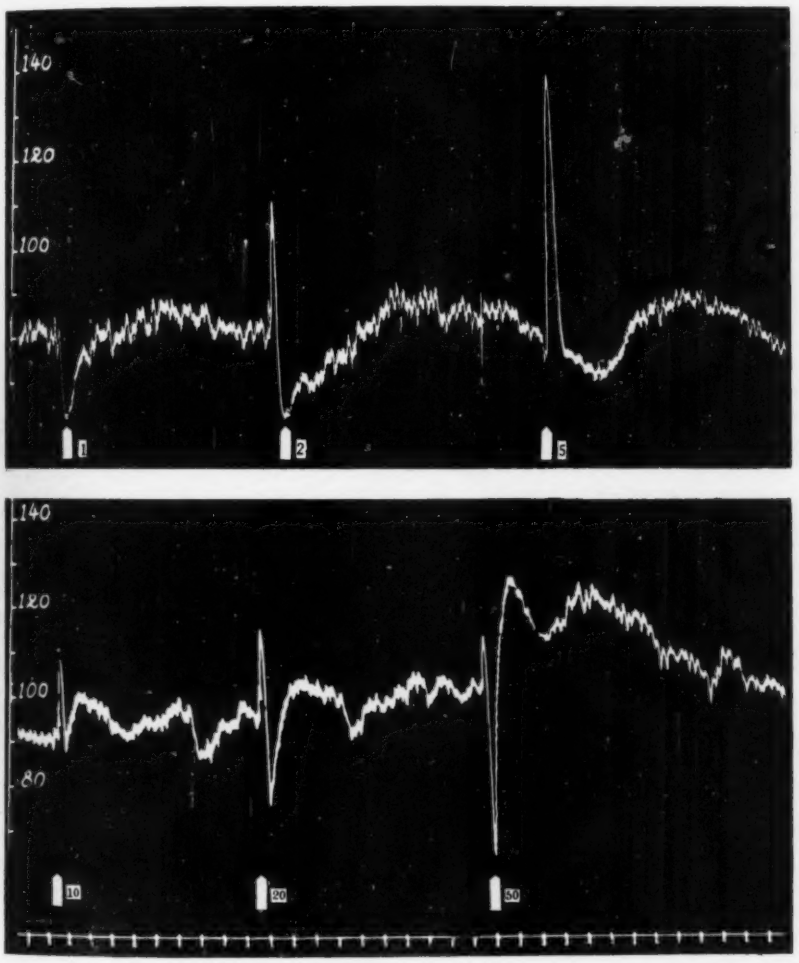

FIG. 2.—Blood pressure records from two rats; urethane-barbitone sodium anaesthesia. Upper record, 190 g. rat, effects of 1, 2 and 5 μg. 5-hydroxytryptamine. Between the 2nd and 3rd dose the drum was stopped for 4 min. Lower record, 200 g. rat, effects of 10, 20 and 50 μg. adenosine. Time 0.5 min.

Dependence on Blood Pressure Level

When the blood pressure of the rat was low, as when the animal had been for some hours under the experimental conditions, or if the injected anaesthetic mixture had a higher proportion of urethane than that mentioned, the effects of 5-hydroxytryptamine were different. Throughout the whole dose range used, even with the smallest effective doses, this substance produced predominantly pressor effects. It follows that a gradual fall of the blood pressure base line from medium levels to low ones was accompanied by an inversion of the effects even of small doses from depressor to pressor. On the contrary, if the blood pressure of the rat was high, as was obtained after anaesthesia with barbiturate only, the pressor components described were less apparent even with large doses of 5-hydroxytryptamine. In contrast to 5-hydroxytryptamine, the blood pressure response to 100 to 200 ng. adrenaline per rat or to 50 to 100 ng. of noradrenaline was of the same order over a wide range of blood pressure

levels (Fig. 3). Like 5-hydroxytryptamine, adenosine showed some change in the response with lowering of the blood pressure base line, but this was not so pronounced.

The dependence of the effects of 5-hydroxytryptamine on blood pressure level has already been outlined for other species by Page (1958), who related it to the influence of neurogenic vascular tone. Whatever may be its cause, this fact was kept in mind when interpreting the actions of antagonists. Thus reduction of depressor effects and potentiation or unmasking of pressor effects after antagonists were deduced only if on several occasions the blood pressure level at the time of injection of 5-hydroxytryptamine (or adenosine) was the same or higher than during the control period. Reduction of pressor effects, on the other hand, could be deduced even if the blood pressure was somewhat lower after the antagonist, but was more difficult to accept if the blood pressure was higher.

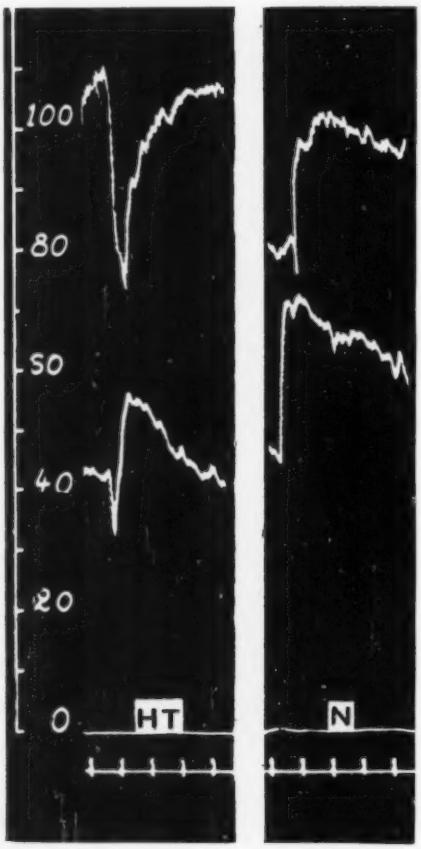

FIG. 3.—Blood pressure records from same rat, 170 g., anaesthetized with urethane. Effects of 1 μg. 5-hydroxytryptamine on left, and 100 ng. noradrenaline on right, at different blood pressures. Time interval between upper and lower records 40 min. Time 0.5 min. Note qualitative change in the response to 5-hydroxytryptamine in contrast to that to noradrenaline.

Actions of Antagonists

In view of the complexity of the effects of 5-hydroxytryptamine no attempt was made to make precise quantitative measurements, but only to determine and describe the main qualitative actions of the antagonists in the anaesthetized rat, breathing spontaneously. All the antagonists studied, with the exception of atropine, had some action against both 5-hydroxytryptamine and catechol amines. In addition, a remarkable feature was that early in the study of each antagonist it became clear that each had a preferential action against either the depressor or pressor component of the effect of 5-hydroxytryptamine; lysergic acid diethylamide and 1 - (3,4-dichlorophenyl) - 2 - isopropylaminoethanol antagonized the depressor response, while dibenamine and 1-benzyl-5-methoxy-2-methyltryptamine antagonized the pressor response.

Lysergic Acid Diethylamide

Small or moderate doses (10 to 40 μg. per 100 g. rat) reduced the depressor effects of 5-hydroxytryptamine, confirming the result of Salmoiraghi, McCubbin, and Page (1957). However, in contrast to the observations of Salmoiraghi *et al.*, antagonism of the pressor components was unusual (two cases out of twelve), and, if present, was slight. On the contrary, in several cases pressor effects were unmasked, that is to say the spike appeared with smaller doses of 5-hydroxytryptamine than were needed before the antagonist and occasionally a dome appeared as well. In some experiments the depressor effect of lysergic acid diethylamide itself might have contributed to the unmasking, but this factor could not wholly account for the phenomenon because unmasking progressed even when the blood pressure tended to return towards the control level (Fig. 4,A). These doses of lysergic acid diethylamide also reduced the pressor effects of 100 to 200 ng. adrenaline per rat and 50 to 100 ng. noradrenaline per rat, but the depressor effects of 5-hydroxytryptamine were more sensitive than the pressor effects of catechol amines, and of the catechol amines adrenaline was more sensitive than noradrenaline to this antagonist. There was also reduction of the depressor effects of adenosine and sometimes unmasking of pressor effects.

Large doses of lysergic acid diethylamide (up to 80 μg. per 100 g. rat) abolished the depressor effects of 5-hydroxytryptamine leaving purely pressor ones (" reversal "), but these pressor effects were reduced. At the same time the effects of these doses of adrenaline were abolished, and those of noradrenaline abolished or greatly reduced. The depressor effects of 50 to 100 ng. isoprenaline per rat and of 100 to 500 ng. acetylcholine per rat were unaffected throughout.

Dihydroergotamine

Small doses of dihydroergotamine (5 to 10 μg. per 100 g. rat) reduced the depressor effect of 5-hydroxytryptamine (Fig. 4,B) and to a less extent the depressor effects of adenosine, but, unlike lysergic acid diethylamide, there was no unmasking of pressor effects. Antagonism of pressor effects of 5-hydroxytryptamine was difficult to determine, owing to the pressor effect usually exerted by dihydroergotamine itself. These small doses of dihydroergotamine were also sufficient to reduce the pressor effects of adrenaline and noradrenaline, which were thus as sensitive to this antagonist as were the depressor effects of 5-hydroxytryptamine. In cases where there were depressor effects of adrenaline these were potentiated.

With moderate doses (15 to 25 μg. per 100 g. rat) antagonism of the depressor effects of 5-hydroxytryptamine and adenosine was increased only slightly, and with large doses (up to 80 μg. per 100 g. rat) no further antagonism occurred. Moderate and large doses, however, reduced further the pressor effects of adrenaline and noradrenaline as well as the depressor effects of isoprenaline, but no marked reduction of the (potentiated) depressor effects of adrenaline occurred. The depressor effects of acetylcholine were unaffected.

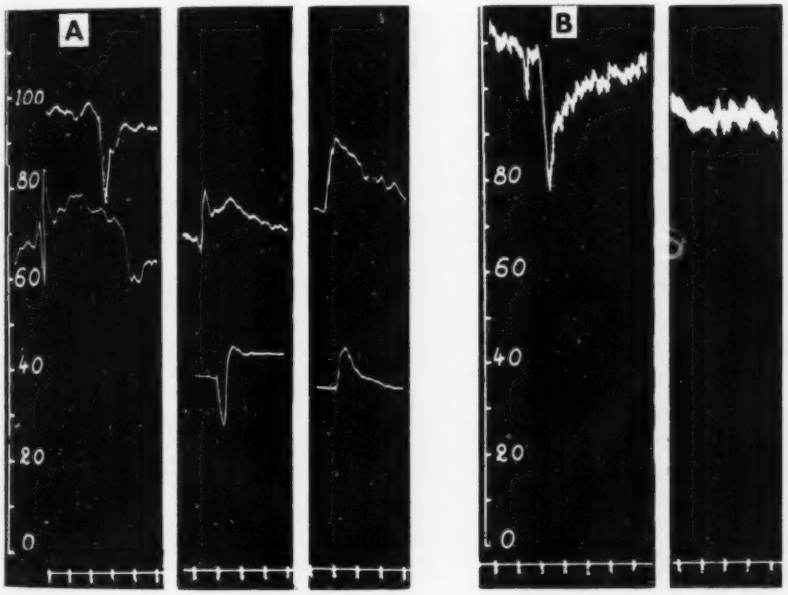

FIG. 4.—Blood pressure records of rats anaesthetized with urethane-barbitone sodium mixture. Time 0.5 min. **A**, Action of lysergic acid diethylamide on effects of 1 μg. 5-hydroxytryptamine. Upper tracings, 175 g. rat, from left to right, injection of 1 μg. 5-hydroxytryptamine 10 min. before, 27 min. after, and 1 hr. 12 min. after 35 μg. lysergic acid diethylamide. Lower tracings, 170 g. rat, injection of 1 μg. 5-hydroxytryptamine 27 min. before, 30 min. after, and 1 hr. 38 min. after 34 μg. lysergic acid diethylamide. Note middle panel shows possible combined actions of lysergic acid diethylamide and of fall of blood pressure; the panel on the right shows further changes possibly related to development of lysergic acid diethylamide antagonism. **B**, Action of dihydroergotamine on effects of 0.5 μg. 5-hydroxytryptamine; 185 g. rat; injection of 0.5 μg. 5-hydroxytryptamine before (left) and 1 hr. 27 min. after (right) dihydroergotamine (two injections, each 9.25 μg., 54 min. and 1 hr. 21 min. after control injection).

1-(3,4-Dichlorophenyl)-2-isopropylaminoethanol

This substance was described by Powell and Slater (1958) as an antagonist of the beta effects of catechol amines. In the present experiments it was found that small doses (125 to 500 μg. per 100 g. rat) markedly antagonized the depressor effects of isoprenaline, abolished a depressor effect of adrenaline if this was present, and reduced somewhat depressor effects of 5-hydroxytryptamine (Fig. 5,A). The pressor effects of adrenaline and noradrenaline were, on the contrary, potentiated and there was sometimes unmasking of pressor effects of 5-hydroxytryptamine. There was no action on the depressor effects of adenosine. Moderate doses produced the same results to a somewhat greater extent, but the depressor effects of adenosine were also slightly reduced with slight unmasking of its pressor effects.

The same actions, but to a still greater extent, occurred with large doses of 1-(3,4-

dichlorophenyl)-2-isopropylaminoethanol (up to 3 mg. per 100 g. rat), the depressor effects of 5-hydroxytryptamine being abolished almost completely, leaving only pressor effects (" reversal "); similar results were obtained with adenosine but to a less degree than with 5-hydroxytryptamine. There was also reduction of the pressor response to adrenaline and noradrenaline. The response to acetylcholine was unaffected. This antagonist therefore, in addition to reducing the depressor effects of catechol amines, also reduced the depressor action of 5-hydroxytryptamine (and to a less extent that to adenosine), and in large doses reduced the pressor reponses to catechol amines as well.

Dibenamine

The three dose levels of dibenamine (from 50 to 400 μg. per 100 g. rat) showed increasing reduction of the pressor effects of 5-hydroxytryptamine (Fig. 5,B) as well as of adrenaline and noradrenaline. The pressor effects of the catechol amines were sometimes abolished, and generally were more sensitive to this antagonist than the pressor effects of 5-hydroxytryptamine, adrenaline being more easily antagonized than noradrenaline. The depressor effects of 5-hydroxytryptamine were unaffected by small doses of dibenamine, but reduced slightly by moderate or large doses. There was no action against the depressor or pressor effects of adenosine.

1-Benzyl-5-methoxy-2-methyltryptamine

Small doses of 1-benzyl-5-methoxy-2-methyltryptamine (100 to 200 μg. per 100 g. rat) reduced the pressor effects of 5-hydroxytryptamine (Fig. 5,C) and the antagonism increased with the dose. With large doses (up to 2.8 mg. per 100 g. rat) the pressor effects of 5-hydroxytryptamine were abolished in all but one case out of nine. The pressor effects of adrenaline and noradrenaline, as well as of adenosine, were affected similarly, but to a less extent. The most sensitive to this antagonist was thus 5-hydroxytryptamine; adrenaline and adenosine were less sensitive and noradrenaline still less. The depressor effects of 5-hydroxytryptamine and adrenaline were increased with small doses of 1-benzyl-5-methoxy-2-methyltryptamine, the former slightly, the latter more markedly (tendency to " reversal "). Moderate and large doses (up to 2.8 mg. per 100 g. rat) of the antagonist had the opposite action on the depressor effects of 5-hydroxytryptamine which were now somewhat reduced, and so were those of isoprenaline, adenosine, and adrenaline; adrenaline being least affected and isoprenaline most affected. The depressor effects of acetylcholine were not antagonized.

Atropine

The administration of atropine (100 μg. per 100 g. rat) produced a marked reduction or abolition of the depressor effects of acetylcholine 250 to 500 ng. per rat, the pressor effects of adrenaline and noradrenaline being unaffected. A further injection of the same dose of atropine caused some reduction of the depressor effects of 5-hydroxytryptamine, and once a slight potentiation of its pressor effects. A particular action shown on adenosine was that the depressor effects

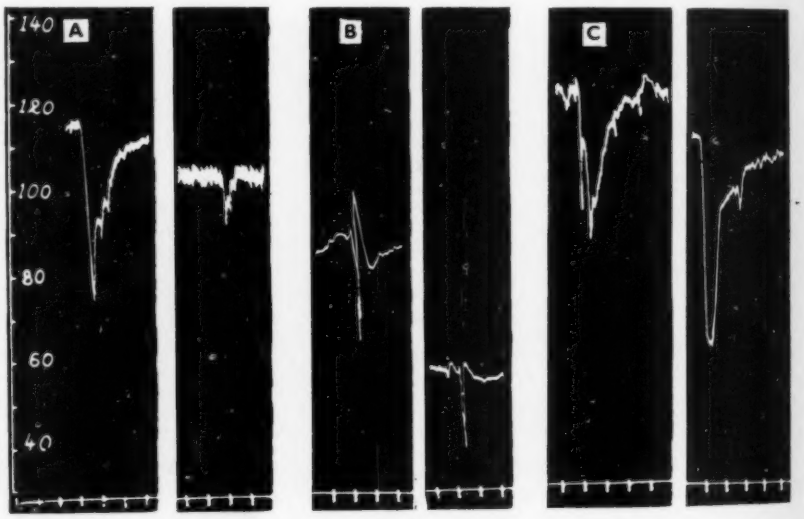

FIG. 5.—Blood pressure records from rats anaesthetized with urethane-barbitone sodium mixture. Time 0.5 min. **A**, Action of 1-3,4-dichlorophenyl)-2-isopropylaminoethanol on effects of 0.5 μg. 5-hydroxytryptamine; 180 g. rat. Injection 11 min. before (left), and 15 min. after (right) 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (360 μg.). **B**, Action of dibenamine on effects of 1 μg. 5-hydroxytryptamine; 195 g. rat. Injection 4 min. before (left) and 30 min. after (right) dibenamine (400 μg.). **C**, Action of 1-benzyl-5-methoxy-2-methyltryptamine on effects of 1 μg. 5-hydroxytryptamine; 180 g. rat. Injection before (left) and 55 min. after (right) 1-benzyl-5-methoxy-2-methyltryptamine (two injections, 360 μg. and 1.8 mg., 9 min. and 34 min. respectively after control injection).

were affected less than the pressor effects, which were potentiated (Fig. 6). A result of this was to make the slope of the dose response curve of adenosine steeper. This action could make the atropinized rat blood pressure preparation a useful one for the assay of adenosine.

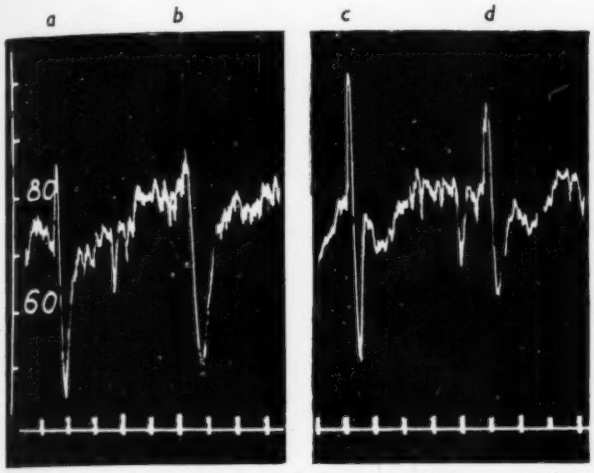

FIG. 6.—Blood pressure records from rat, 200 g., anaesthetized with urethane-barbitone sodium mixture. Action of atropine on effects of adenosine and 5-hydroxytryptamine. Responses to 20 μg. adenosine and 1 μg. 5-hydroxytryptamine alone at (a) and (b) respectively, and 1 hr. after atropine (two injections, each 200 μg., 5 min. and 35 min. after control injections) at (c) and (d).

The different patterns of antagonism shown are summarized in the Table, which embodies the salient features by means of a rough quantitative code. In addition to these results some further pecularities of lysergic acid diethylamide are noteworthy: (i) the antagonistic action of small or moderate doses required 0.5 to 1 hr. to develop fully, contrary to the other antagonists whose action appeared to be fully developed within much shorter times; this characteristic of lysergic acid diethylamide is illustrated in Fig. 4,A; (ii) a particular antagonistic action by lysergic acid diethylamide, once developed, appeared to persist for several hours; this was not the case for 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol; in some experiments the action of small or moderate doses of the latter wore off completely in 45 to 60 min.; (iii) the dose range over which the whole sequence of antagonisms developed in each rat was smallest (2 to 4 fold) for lysergic acid diethylamide, much greater for 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol (25 fold), the dose ranges of the other antagonists being intermediate (dibenamine 6 to 8 fold, dihydroergotamine 8 to 10 fold, 1-benzyl-5-methoxy-2-methyltryptamine 10 to 20 fold); (iv) the differences of sensitivity of the various agonist drugs to each antagonist were more clearly marked in the case of lysergic acid diethylamide than the others.

TABLE

RELATIVE SENSITIVITY OF DRUGS TO ANTAGONISTS

The pressor or depressor responses of the rat blood pressure to various agonists were not affected (**0**), or diminished by large (**1**), moderate (**2**) or small (**3**) doses of anta onist or the antagonism was doubtful (**?**). The antagonists revealed unma king (**U**) or potentiation (**P**) of effects with small or moderate doses, sometimes followed by antagonism (**U→1**), (**P→1**) with large doses of antagonist.

Antagonist	No. of Expts.	Catechol Amines				5-Hydroxy-tryptamine		Adenosine		Acetyl-choline
		Depressor (Beta)		Pressor (Alpha)		De-pressor	Pressor	De-pressor	Pressor	De-pressor
		Isopren-aline	Adren-aline	Adren-aline	Norad-renaline					
Lysergic acid diethylamide ..	(12)	0		2	2	3	U→1	2	U	0
Dihydroergotamine	(5)	1	P	3	3	3	?	2	0 or U	0
1-(3,4-Dichlorophenyl)-2-isopropylaminoethanol ..	(8)	3	3	P→1	P→1	2	U	1	U	0
Dibenamine	(5)			3	3	0 or 1	2	0	0	
1-Benzyl-5-methoxy-2-methyltryptamine	(9)	2	P→1	2	2	P→1	3	1	2	0

Discussion

The mixed effects of 5-hydroxytryptamine on the rat's blood pressure have been described by Salmoiraghi *et al.* (1956). Their results were substantially confirmed in our experiments for the particular dose range (2 to 5 μg. per rat) used by those authors. The study of a wider range of doses, however, disclosed a pattern of qualitative effects from mainly depressor responses resembling those in cats and rabbits to responses in which pressor components predominated as in dogs.

Part of the depressor effects of 5-hydroxytryptamine could be of parasympathetic origin because it was antagonized, though only to some extent, by atropine in maximally tolerated doses which completely inhibited the effects of injected acetylcholine. A vagal component of the effects of 5-hydroxytryptamine on the blood pressure of rats has already been shown by Salmoiraghi *et al.* (1956).

Part of both depressor and pressor effects of 5-hydroxytryptamine might also be of sympathetic origin in that all of the antagonists studied had some action against both 5-hydroxytryptamine and the catechol amines. In particular it would be tempting to draw conclusions as to the mediation of the effects of 5-hydroxytryptamine through catechol amines, depressor effects through hypotensive catechol amines, owing to the almost complete antagonism by 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol, a selective beta inhibitor, and pressor effects through hypertensive catechol amines, owing to the antagonism by dibenamine. In this hypothesis the relative resistance of liberated substances compared with injected ones would have to be considered. The relationships between the effects of 5-hydroxytryptamine and the catechol amines appear, however, to be more complex, as not only corresponding effects but also opposite effects of these substances can be similarly antagonized by some agents, for example, the depressor effects of 5-hydroxytryptamine and the pressor effects of catechol amines by lysergic acid diethylamide and dihydroergotamine. This mixed antagonism shows that antagonism against corresponding effects does not necessarily mean the interruption at a particular point of a process of mediation. It can also be a clue to some relationship of another kind between 5-hydroxytryptamine and the catechol amines, for example, some common structural feature in their receptors. In this connexion one may recollect that on isolated organs, for example, the rat's uterus, typical inhibitors of catechol amines like dihydroergotamine and dibenamine also have a powerful antagonistic action against 5-hydroxytryptamine (Erspamer, 1953; Gaddum and Hameed, 1954), whose excitatory effects cannot be mediated by catechol amines which, under those conditions, are inhibitory (Gaddum, Peart, and Vogt, 1949). There are two further pieces of evidence that the depressor response to 5-hydroxytryptamine can be only partly, if at all, of sympathetic origin, namely, that this response was diminished by atropine and only partly affected even by large doses of dihydroergotamine.

It was reported by Salmoiraghi *et al.* (1957) that 10 to 50 μg. lysergic acid diethylamide per rat reduced the depressor effects of 5-hydroxytryptamine but failed to affect the response to 1 μg. of noradrenaline. In our experiments the pressor effects of 50 to 100 ng. noradrenaline per rat were also reduced and with larger doses of lysergic acid diethylamide almost abolished, so that the antagonist did not show an absolute specificity against 5-hydroxytryptamine. In fact none of the antagonists studied can be considered to possess a high degree of specificity. But it is possible to distinguish two substances (lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine) which were relatively selective against 5-hydroxytryptamine, and two other substances (1-(3,4-dichlorophenyl)-2-isopropylaminoethanol and dibenamine) whose antagonism was directed rather more against the catechol amines. Dihydroergotamine was as powerful against 5-hydroxytryptamine as against the catechol amines and so occupied an intermediate position. 5-Hydroxytryptamine and adenosine, on the other hand, showed a common susceptibility or resistance to all the antagonists in this study, but adenosine in most cases was affected less than 5-hydroxytryptamine, and the development of effects which the two drugs showed on increasing the dose was in opposite directions. After most of the antagonists differences were diminished, but in the particular case of 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol it was emphasized. In consequence, to distinguish between 5-hydroxytryptamine and adenosine in a solution containing one of them it might be useful to compare their effects before and after the administration of 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol.

When classified according to their actions against the two effects of 5-hydroxytryptamine the antagonists studied fall into two main groups. One group containing lysergic acid diethylamide and 1-(3,4-dichlorophenyl)-2-isopropylaminoethanol antagonized preferentially the depressor effects of 5-hydroxytryptamine, while the other group containing 1-benzyl-5-methoxy-2-methyltryptamine and dibenamine antagonized prefer-

entially its pressor effects. It was difficult to classify dihydroergotamine. The compound antagonized the depressor effects of 5-hydroxytryptamine, but its action, if any, on the pressor effects of 5-hydroxytryptamine was obscured by its own pressor action. It is noteworthy that the two selective antagonists of 5-hydroxytryptamine already mentioned (lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine) belong, according to this classification, to different groups. Lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine differed from the selective antagonists of catechol amines in that after antagonism of the susceptible effect (depressor in the former case and pressor in the latter) they first potentiated the resistant effect, and finally, in doses 4 to 10 times as large, antagonized this as well. They thus showed a capacity for antagonizing all the effects of 5-hydroxytryptamine. The preferential actions of lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine against different effects of 5-hydroxytryptamine agree well with the relative inefficacy of lysergic acid diethylamide in dogs (Salmoiraghi *et al.*, 1957), in which 5-hydroxytryptamine is mainly pressor, and with the efficacy of 1-benzyl-5-methoxy-2-methyltryptamine in the same species (Shaw and Woolley, 1956) as well as its weak action in rabbits (Jacob and Cugurra, 1960) where depressor effects predominate.

The actions of lysergic acid diethylamide and 1-benzyl-5-methoxy-2-methyltryptamine, antagonizing respectively the depressor and pressor actions of 5-hydroxytryptamine, bear some resemblance to those of the two kinds of antagonists of acetylcholine. Gaddum and Hameed (1954) put forward the theory that, as in the case of acetylcholine, there are at least two types of receptor with which 5-hydroxytryptamine combines. However, in view of the complexity of effects in the whole anaesthetized and spontaneously respiring rat and without more information on the modes of action of the antagonists, any attempt to correlate the types of antagonism here described with kinds of receptors would be only speculative.

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THE 5-HYDROXYTRYPTAMINE CONTENT OF MOUSE BRAIN AND WHOLE MICE AFTER TREATMENT WITH SOME DRUGS AFFECTING THE CENTRAL NERVOUS SYSTEM

BY

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A number of drugs were examined for their ability to change the concentration of 5-hydroxytryptamine in mouse brain and in whole mice treated with 5-hydroxytryptophan. After β-phenyl-*iso*propylhydrazine or iproniazid, two inhibitors of monoamine oxidase, the brain 5-hydroxytryptamine rose to a maximum value in 8 hr., after which it declined, although a slight rise remained for as long as 6 days. Dose-effect relationships, determined 6 hr. after administration, showed β-phenyl-*iso*propylhydrazine to be approximately 60 times as effective as iproniazid in raising the brain 5-hydroxytryptamine. When mice were given 5-hydroxytryptophan and the amine content of the whole mice estimated, pretreatment with β-phenyl*iso*propylhydrazine increased their 5-hydroxytryptamine content whereas pretreatment with iproniazid did not change it. The concentration of the amine in mouse brain and in whole mice was lower after reserpine, but was raised when reserpine and β-phenyl*iso*propylhydrazine were given together. A small rise in brain 5-hydroxytryptamine was found after chlorpromazine ; when chlorpromazine was given with iproniazid, however, the resulting increase was less than that found after iproniazid alone. Brain 5-hydroxytryptamine was unaltered after prolonged treatment with morphine.

Monoamine oxidase is widely distributed in animal tissues and is an important route for the inactivation of 5-hydroxytryptamine (Davison, 1958 ; Keglević, Supek, Kveder, Iskrić, Kečkeš, and Kisić, 1959). Effective inhibitors of monoamine oxidase should therefore retard the inactivation of the amine *in vivo*, but this is only partially true for l-*iso*nicotinyl-2-*iso*propylhydrazine (iproniazid), the most widely used inhibitor of monoamine oxidase. Although iproniazid is a strong and fairly specific inhibitor of this enzyme in homogenates (Zeller, Barsky, Fouts, Kircheimer, and Van Orden, 1952 ; Zeller, Barsky, and Berman, 1955), it has little effect on the inactivation of 5-hydroxytryptamine in tissue slices or in intact mice (Udenfriend, Weissbach, and Bogdanski, 1957). More recently, Horita (1958) has reported β-phenyl*iso*propylhydrazine to be a potent inhibitor of monoamine oxidase. In the present experiments, both β-phenyl*iso*propylhydrazine and iproniazid were examined as inhibitors of 5-hydroxytryptamine inactivation in intact mice.

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5-Hydroxytryptamine is present in the nervous system of a wide variety of species (Amin, Crawford, and Gaddum, 1954 ; Correale, 1956), and this has led to much interest in possible relationships between 5-hydroxytryptamine and drugs which affect brain function (Gaddum, 1954 ; Woolley and Shaw, 1954 ; Brodie, Pletscher, and Shore, 1955 ; Paasonen and Vogt, 1956 ; Pletscher, 1957). Chlorpromazine, reserpine and morphine, three drugs with actions on the central nervous system, have therefore been included as test substances in some of the present experiments.

METHODS

Materials.—Male albino mice, of body weight 10 to 20 g., were maintained on cube diet 41B with free access to water. The drugs used were chlorpromazine hydrochloride, β-phenyl*iso*propylhydrazine hydrochloride, iproniazid phosphate, reserpine, morphine sulphate, 5-hydroxytryptamine creatinine sulphate, (±)5-hydroxytryptophan, tryptamine hydrochloride, and (±)tryptophan. Quantities of tryptamine and 5-hydroxytryptamine have been expressed in terms of the base, but quantities of β-phenyl*iso*propylhydrazine, iproniazid, morphine and chlorpromazine refer to the

salts used. Drugs were freshly dissolved in distilled water, except reserpine, which was dissolved in a few drops of glacial acetic acid, diluted with 50 volumes of a mixture of equal parts of propylene glycol and ethyl alcohol, and made up to 100 volumes with distilled water. The stock solution of reserpine was diluted with distilled water immediately before use. Injections were given intraperitoneally in a dose volume of 0.1 ml./10 g. mouse, with the exception of 5-hydroxytryptophan, which was given in a dose volume of 0.2 ml./10 g. mouse.

Morphine Injected Mice.—Mice were injected daily with an increasing dose of morphine, under the supervision of Dr. S. J. Corne. The dose was increased as follows: 12.5 mg./kg. (10 days), 25 mg./kg. (21 days), 50 mg./kg. (7 days) and 100 mg./kg. (3 days). The final injection of morphine was given one hour before killing the mice and extracting 5-hydroxytryptamine.

Extraction of 5-Hydroxytryptamine.—Whole brains were dissected from mice which had been killed and bled out from the neck. The pooled brains from three mice were cut up finely with a scalpel on a glass slide and were extracted with 4 volumes of acetone (Analar). Next day, the acetone extract was separated from the residue and the residue was re-extracted overnight with 4 volumes of acetone. The second acetone extract was separated from the residue and the two extracts combined.

For the extraction of whole mice the acetone was acidified by the addition of 10 ml. N HCl/l. of acetone. Two mice were killed and homogenized with 4 g. sodium chloride and 4 volumes of acidified acetone. Next day the homogenate was filtered, and the residue extracted a second time overnight with 4 volumes of acidified acetone. On the following day the acetone extract was separated by filtration, and the residue on the filter paper washed with 30 ml. acidified acetone. The acetone extracts and washings were combined.

All extracts were kept at 0 to 4°. Just prior to the bioassay, an aliquot of acetone extract was evaporated to dryness at an external temperature of 35° and the residue re-extracted with a convenient volume of 0.9% sodium chloride.

Estimation of 5-Hydroxytryptamine. — The 5-hydroxytryptamine content of the saline extracts was determined on the isolated uterus of an oestrous rat. The uterus was suspended in a 15 ml. bath of oxygenated de Jalon solution at 29°. Atropine sulphate at a concentration of 10^{-6} was added to the de Jalon solution to increase the specificity of the preparation, which always responded to 0.02 to 0.03 μg. 5-hydroxytryptamine.

Rat Fundus.—The rat fundus preparation was made as described by Vane (1957). The fundus strip was suspended in a 10 ml. bath of oxygenated Tyrode solution at 37°, and contractions were recorded from a pendular lever (Paton, 1957). Atropine sulphate was added to the Tyrode solution to give a concentration of 10^{-6}.

Paper Chromatography.—Acetone extracts were evaporated to dryness at 35°, and the residue extracted with 0.25 ml. N HCl and 3 ml. ether. After shaking, the ether layer was discarded, and the aqueous phase transferred to an evaporating dish with 2 ml. alcohol. The extract was concentrated to 0.1 to 0.2 ml. and applied to a sheet of Whatman No. 1 paper together with marker spots of tryptamine, 5-hydroxytryptamine and tryptophan. Chromatograms were developed by the descending technique with *iso*propanol/ammonia (880)/water (20:1:2), or with *n*-butanol/acetic acid glacial/water (12:3:5). Occasionally two-way chromatograms were prepared with the same two solvent systems. All chromatograms were run for 5.5 to 7 hr. at a room temperature of 25.5 to 29.5°. Indoles were detected on the dried chromatograms by spraying with a 2% solution of *p*-dimethylaminobenzaldehyde in N HCl (Ehrlich's reagent), followed by heating at 80 to 85° for 3 to 5 min.

RESULTS

Identification of 5-Hydroxytryptamine in Extracts.—When bromolysergic acid diethylamide at a concentration of 10^{-7} was included in the de Jalon solution, the uterine responses to both 5-hydroxytryptamine and extracts were almost always abolished. Occasionally a brain extract still had a very slight stimulant action indicating the presence of a trace of interfering substance, but it was clear that the extracts reacted mainly with tryptamine receptors (Gaddum, 1953; Gaddum and Hameed, 1954).

Tryptamine has been identified in guinea-pig brain after administration of tryptophan and iproniazid (Hess, Redfield, and Udenfriend, 1959). Tryptamine and 5-hydroxytryptamine both react with tryptamine receptors and both are substrates of monoamine oxidase. The extracts were therefore examined for tryptamine, since this might have accounted for a significant part of their stimulant activity on the uterus. In extracts of mouse brains and whole mice 5-hydroxytryptamine was identified chromatographically as a blue spot with an R_F of 0.49 in butanol/acetic, and an R_F of 0.48 in *iso*propanol/ammonia. Tryptamine was not detected on these chromatograms. Even so, it seemed possible that tryptamine was present in small amounts. Tryptamine and 5-hydroxytryptamine can now be differentiated biologically, since Vane (1959) has shown that inhibitors of monoamine oxidase potentiate the response of the rat fundus preparation to tryptamine but not to 5-hydroxytryptamine. Tryptamine, 5-hydroxytryptamine, and brain extracts of normal mice and of mice treated with β-phenyl*iso*propylhydrazine were tested on rat fundus preparations both before and after the addition of iproniazid 10^{-5}. After

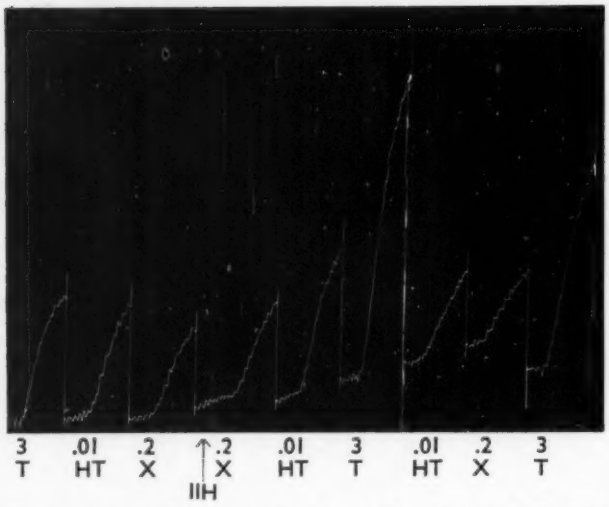

FIG. 1.—Rat fundus. 10 ml. bath. Interval 5 min. T, tryptamine (μg.). HT, 5-hydroxytryptamine (μg.). X, brain extract from control mice (ml.). From IIH, iproniazid 10^{-5} was present in the Tyrode solution. Iproniazid potentiated the response to tryptamine, but did not potentiate the responses to 5-hydroxytryptamine or extract.

iproniazid, the response of a fundus to tryptamine was potentiated whereas the contractions produced by 5-hydroxytryptamine and the extracts remained relatively unaffected (Fig. 1). This was consistent with the view that the active material in the extracts was 5-hydroxytryptamine rather than tryptamine.

The Effect of Drugs on Uterine Response to 5-Hydroxytryptamine.—The following drugs were added to the organ bath at the same time as 5-hydroxytryptamine: 0.5 μg. reserpine, 2 μg. chlorpromazine, 30 μg. iproniazid, 10 μg. β-phenyl-*iso*propylhydrazine, and 100 μg. morphine. The response to 5-hydroxytryptamine was reduced after chlorpromazine but was unaffected by the other drugs. When brain extracts of mice treated with chlorpromazine were tested there was no inhibition of the response to 5-hydroxytryptamine, however, showing that the extracts contained insufficient chlorpromazine to affect the uterus.

Recovery of 5-Hydroxytryptamine.—The extraction procedures adopted were similar to those of Correale (1956). The recovery of 5-hydroxytryptamine was tested by the addition of 0.5 μg. 5-hydroxytryptamine to three brain extracts (1 g. of brain approximately) and 200 μg. 5-hydroxytryptamine to two whole mice (30 g. approximately). In 5 determinations a mean of $73.2 \pm 2.0\%$ of the added 5-hydroxytryptamine was recovered from mouse brain, and, in 4 determinations, $64.5 \pm 5.2\%$ from whole mice. The recovery of added 5-hydroxytryptamine from whole mice remained unchanged at $63.0 \pm 7.6\%$ (4 determinations) when the mice were injected with β-phenyl*iso*propylhydrazine (100 mg./kg.). Differences in the 5-hydroxytryptamine content of the extracts may be taken to represent differences in the 5-hydroxytryptamine content of the mice, since β-phenyl*iso*propylhydrazine, an effective inhibitor of 5-hydroxytryptamine inactivation *in vivo*, did not alter the recovery of 5-hydroxytryptamine from the mice. Although Correale reported the recovery of 90–100% of added 5-hydroxytryptamine, other workers have obtained recoveries similar to those reported here, e.g. Twarog and Page (1953) recovered 60% of the added 5-hydroxytryptamine after extraction with acetone, and Welsh and Moorhead (1959) recovered 70% by a butanol extraction procedure.

The results given below have not been corrected for the loss of 5-hydroxytryptamine in the extraction procedure. A result has been taken as significant when P was less than 0.05.

TABLE I

INCREASES IN 5-HYDROXYTRYPTAMINE CONCENTRATION IN MOUSE BRAIN 1 TO 6 DAYS AFTER β-PHENYL*ISO*PROPYL-HYDRAZINE AND IPRONIAZID

In 7 control mice the brain 5-hydroxytryptamine concentration was 0.624 ± 0.099 μg./g. brain.

Drug	Days after Administration of Drug	Mean Increase in 5-Hydroxytryptamine (μg./g. Brain ± S.E.). No. of Determinations in Brackets	Significance of Mean from Zero (P)
β-Phenyl*iso*-propyl-hydrazine. (20 mg./kg.)	1	$+0.486 \pm 0.050$ (2)	<0.001
	2	$+0.063 \pm 0.050$ (2)	<0.25, >0.1
	3	$+0.202 \pm 0.050$ (2)	<0.001
	4	$+0.169 \pm 0.045$ (2)	<0.001
	5	$+0.176 \pm 0.045$ (2)	<0.001
	6	$+0.128 \pm 0.045$ (2)	<0.01, >0.005
Iproniazid (300 mg./kg.)	1	$+0.379 \pm 0.050$ (2)	<0.001
	2	$+0.146 \pm 0.050$ (2)	<0.005, >0.0025
	3	$+0.311 \pm 0.050$ (2)	<0.001
	4	$+0.175 \pm 0.045$ (2)	<0.001
	5	$+0.187 \pm 0.045$ (2)	<0.001
	6	$+0.165 \pm 0.045$ (2)	<0.0025, >0.001

5-Hydroxytryptamine in Mouse Brain.—Brain 5-hydroxytryptamine concentrations after administration of drugs have been expressed as differences between treated and control mice of the same experiment, the control values being given at the head of each table.

The effect of a single injection of β-phenyl*iso*propylhydrazine (20 mg./kg.) or of iproniazid (300 mg./kg.) on mouse brain 5-hydroxytryptamine was examined over a period of days (Table I). One day after β-phenyl*iso*propylhydrazine and iproniazid, increases in brain 5-hydroxytryptamine were 0.486 μg./g. and 0.379 μg./g. respectively, from a control value of 0.624 μg./g. Even 6 days after these monoamine oxidase inhibitors, small but significant increases in brain 5-hydroxytryptamine were found.

Increases in mouse brain 5-hydroxytryptamine concentration after β-phenyl*iso*propylhydrazine (100 mg./kg.) and iproniazid (300 mg./kg.) were also investigated at more closely spaced time intervals over a period of 24 hr. (Fig. 2). The maximum increases in amine were found 8 hr. after administration of either compound. However, for practical reasons the effect of different doses of β-phenyl*iso*propylhydrazine and iproniazid in raising the brain 5-hydroxytryptamine was investigated after 6 hr. (Fig. 3). The dose-effect curve for iproniazid had a flatter slope and a lower maximum than the corresponding curve for β-phenyl*iso*propylhydrazine. It was not possible therefore to give an accurate ratio of the potencies for these two compounds. However, the effects of 5 mg./kg. β-phenyl*iso*propylhydrazine and 300 mg./kg. iproniazid were of the same order, since they increased the brain 5-hydroxytryptamine by 0.347 μg./g. and by 0.389 μg./g., respectively.

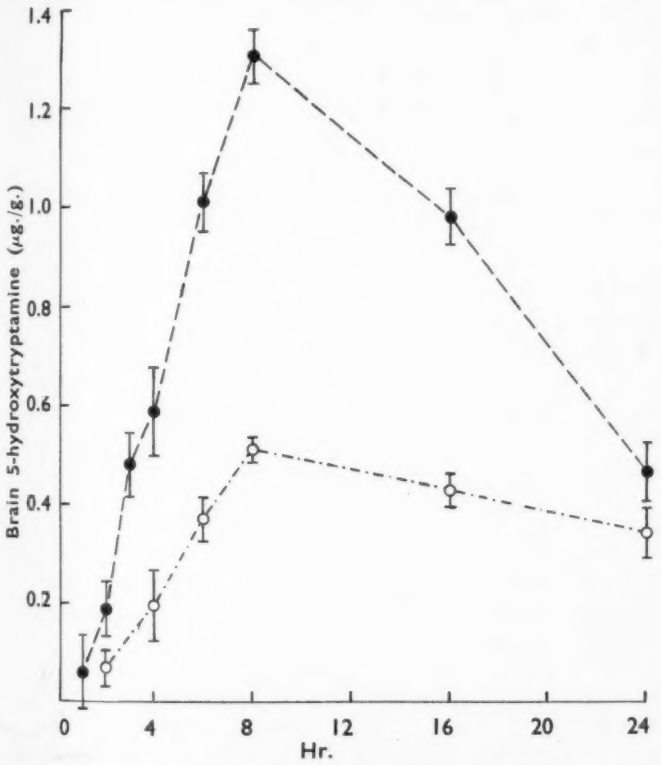

FIG. 2.—Increases in brain 5-hydroxytryptamine in mice after treatment with β-phenyl*iso*propylhydrazine (100 mg./kg.), marked ● – – – ●, or iproniazid (300 mg./kg.), marked O- · · · -O. The mean value for 5-hydroxytryptamine in control brains was 0.497±0.032 μg./g. (17 determinations). Ordinate, increase in brain 5-hydroxytryptamine (μg./g.). Abscissa, interval between injection of compound and extraction of 5-hydroxytryptamine (hr.). Each point is a mean value±S.E.

Table II shows differences in the 5-hydroxytryptamine concentration of mouse brain 6 hr. after the intraperitoneal injection of various drugs. The concentration of amine was lower after reserpine (2 mg./kg.), was higher after β-phenyl*iso*propylhydrazine (20 mg./kg.), and was increased by the combination of these two drugs at the same dose levels. When all the data were combined as in Table II, the rise in 5-hydroxytryptamine after β-phenyl*iso*propylhydrazine was higher than that after reserpine plus β-phenyl*iso*propylhydrazine. However, when the comparison was restricted to results determined in parallel, the difference between the two groups was not significant ($P>0.2$). A small but significant rise was found in each of 9 experiments after chlorpromazine (20 mg./kg.), but the increase in brain 5-hydroxytryptamine after chlorpromazine plus iproniazid was significantly less than the increase found after iproniazid alone ($P<0.02$, >0.01). No significant difference in 5-hydroxytryptamine concentration was found between the brains of control mice and mice treated with morphine.

5-Hydroxytryptamine in Whole Mice Injected with 5-Hydroxytryptophan.—Table III shows the effect of some drugs on the 5-hydroxytryptamine extracted from mice injected with 5-hydroxytryptophan. All mice received 5-hydroxytryptophan (150 mg./kg.), and 3 hr. later were killed and extracted for

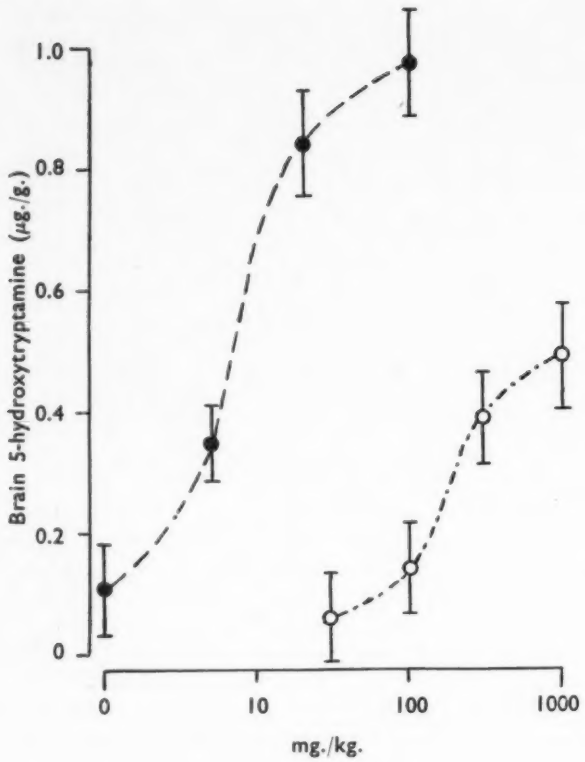

FIG. 3.—Increases in brain 5-hydroxytryptamine in mice 6 hours after varying doses of β-phenyl*iso*propylhydrazine, marked ●---●, and iproniazid, marked O-·-·-O. The mean value for 5-hydroxytryptamine in control brains was 0·449±0·029 μg./g. (10 determinations). Ordinate, increases in brain 5-hydroxytryptamine (μg./g.). Abscissa, doses of β-phenyl*iso*propylhydrazine and iproniazid (mg./kg.), plotted on a logarithmic scale. Each point is a mean value±S.E.

5-hydroxytryptamine. Drugs tested in the mice were administered intraperitoneally at specified periods before injecting the mice with 5-hydroxytryptophan. Treatment with reserpine (2 mg./kg.) lowered the 5-hydroxytryptamine concentration in mice; in animals treated with β-phenyl*iso*propylhydrazine (100 ml./kg.), it was increased above control levels, but values after treatment with iproniazid (300 mg./kg.) were not significantly different from the controls. 5-Hydroxytryptamine was also increased when β-phenyl*iso*propylhydrazine and reserpine were injected together; indeed, in mice injected with β-phenyl*iso*propylhydrazine plus reserpine it was not significantly different from β-phenyl*iso*propylhydrazine treated mice (P>0.2).

TABLE II

CHANGES IN 5-HYDROXYTRYPTAMINE CONCENTRATION IN MOUSE BRAIN 6 HOURS AFTER INTRAPERITONEAL INJECTION OF VARIOUS DRUGS

In 18 control mice the brain 5-hydroxytryptamine concentration was 0·478±0·027 μg./g. brain.

Drug	Dose (mg./kg., i.p.)	Changes in 5-Hydroxytryptamine (μg./g. brain±S.E.). No. of Determinations in Brackets	Significance of Mean from Zero (*P*)
Reserpine	2	−0·264±0·012 (6)	<0·001
Reserpine + β-phenyl*iso*propylhydrazine	2 20	+0·512±0·012 (6)	<0·001
β Phenyl*iso*propylhydrazine	20	+0·746±0·076 (12)	<0·001
Iproniazid	300	+0·445±0·057 (11)	<0·001
Iproniazid + Chlorpromazine	300 20	+0·286±0·065 (9)	<0·005, >0·002
Chlorpromazine	20	+0·108±0·021 (9)	<0·001
Morphine	(See methods)	−0·001±0·025 (3)	>0·9

DISCUSSION

Both β-phenyl*iso*propylhydrazine and iproniazid caused an accumulation of 5-hydroxytryptamine in mouse brain. Between 2 and 8 hr. after β-phenyl*iso*propylhydrazine (100 mg./kg.) or iproniazid (300 mg./kg.), the rise of 5-hydroxytryptamine in mouse brain was very rapid, 1.12 μg./g. brain accumulating after β-phenyl*iso*propylhydrazine. The accumulation of 5-hydroxytryptamine in brain after the monoamine oxidase inhibitors showed that it was synthesized at a high rate; it was calculated that the normal brain content accumulated in about 2.5 hr. after β-phenyl*iso*propylhydrazine. The amine reached its maximum concentration in brain 8 hr. after β-phenyl*iso*propylhydrazine or iproniazid and then declined. This decline may have been due to re-activation or re-formation of brain monoamine oxidase. Complete regeneration of brain

TABLE III

THE EFFECT OF SOME DRUGS ON 5-HYDROXYTRYPTAMINE LEVELS IN WHOLE MICE TREATED WITH 5-HYDROXYTRYPTOPHAN

All mice received 5-hydroxytryptophan (150 mg./kg.) 3 hr. before extraction of 5-hydroxytryptamine. In 8 control mice the concentration of 5-hydroxytryptamine was 3·91±0·26 μg./g. mouse.

Drug (hr. Before Injection of 5-Hydroxy-tryptophan)	Dose (mg./kg., i.p.)	5-Hydroxy-tryptamine (μg./g. Mouse ±S.E.). No. of Determinations in Brackets	Significance of Difference Between Means of Treated and Control Mice (*P*)
Reserpine (3 hr.)	2	2·76±0·25 (3)	<0·05, >0·02
Reserpine (3 hr.)+ β-phenyl*iso*propyl-hydrazine (1 hr.)	2 100	6·37±0·75 (3)	<0·0025, >0·001
β-Phenyl*iso*propyl-hydrazine (1 hr.)	100	7·73±0·64 (6)	<0·001
Iproniazid (1 hr.)	300	3·94±0·68 (4)	>0·90

monoamine oxidase was a lengthy process, however, since the brain 5-hydroxytryptamine was still slightly raised after 6 days.

When different doses of β-phenyl*iso*propylhydrazine and iproniazid were compared for their ability to raise the brain 5-hydroxytryptamine concentration, 6 hr. after administration of drug, β-phenyl*iso*propylhydrazine was found to be about 60 times as effective as iproniazid, since 5 mg./kg. β-phenyl*iso*propylhydrazine and 300 mg./kg. iproniazid both produced a rise of 0.3 to 0.4 μg. 5-hydroxytryptamine/g. brain. A similar order of effectiveness was found for these two compounds when they were examined as *in vitro* inhibitors of monoamine oxidase (Udenfriend, Witkop, Redfield, and Weissbach, 1958). The present results therefore support the view that the rise in 5-hydroxytryptamine in brain after β-phenyl*iso*propylhydrazine and iproniazid was due to inhibition of brain monoamine oxidase.

Log dose-effect curves were obtained by plotting increases in brain 5-hydroxytryptamine against doses of β-phenyl*iso*propylhydrazine or iproniazid. Sigmoid curves were obtained for both compounds, but the curve for iproniazid had a flatter slope and a lower maximum than the curve for β-phenyl*iso*propylhydrazine. The lower maximum of its curve suggested that iproniazid might inhibit both formation and inactivation of 5-hydroxytryptamine in the brain. According to Davison (1956), a metabolite of iproniazid inhibits enzymes requiring pyridoxal phosphate. This supports the present interpretation of the log dose-effect curve for iproniazid, since pyridoxal phosphate is a co-enzyme of the decarboxylase which forms brain 5-hydroxytryptamine.

Inhibition of 5-hydroxytryptamine inactivation by β-phenyl*iso*propylhydrazine must be widespread in the tissues of the mouse, since the 5-hydroxytryptamine content of animals injected with 5-hydroxytryptophan was increased after treatment with β-phenyl*iso*propylhydrazine (100 mg./kg.). This was a severe test of the effect of β-phenyl*iso*propylhydrazine, since the substrate was presented to the enzyme *in vivo*; iproniazid tested in the same way was without effect at 300 mg./kg.

After reserpine (2 mg./kg.), 5-hydroxytryptamine was reduced by 63% in mouse brain and by 30% in whole mice. When β-phenyl*iso*propylhydrazine was given with reserpine, the 5-hydroxytryptamine concentrations of mouse brain and whole mice were raised, suggesting that monoamine oxidase is involved in the disappearance of 5-hydroxytryptamine after reserpine. The 5-hydroxytryptamine content of mice injected with 5-hydroxytryptophan and β-phenyl*iso*propylhydrazine remained unaltered when the mice were pretreated with reserpine. This showed that reserpine (2 mg./kg.) had no appreciable effect on the decarboxylation of 5-hydroxytryptophan, which is in agreement with Brodie, Tomich, Kuntzman and Shore (1957). The difference between the increase in brain 5-hydroxytryptamine found after reserpine plus β-phenyl*iso*propylhydrazine and that found after β-phenyl*iso*propylhydrazine alone was not significant when the comparison was made between results determined in parallel.

The increase in brain 5-hydroxytryptamine concentration found after iproniazid plus chlorpromazine was significantly less than the increase found after iproniazid alone. This suggested a depression of 5-hydroxytryptamine formation after chlorpromazine. According to West (1958), chlorpromazine inhibits the 5-hydroxytryptophan decarboxylase of rat kidney *in vivo*. Such a decarboxylase inhibitor would be expected to cause a fall in brain 5-hydroxytryptamine within

a few hours, since the rate of formation of 5-hydroxytryptamine in brain has been shown to be high. After chlorpromazine, however, 5-hydroxytryptamine concentration in the brain was slightly raised above the corresponding control values. The reason for the small rise in brain 5-hydroxytryptamine after chlorpromazine is not understood. Possibly chlorpromazine inhibited some route of 5-hydroxytryptamine inactivation. According to Weissbach (1958), 5-hydroxytryptamine can be inactivated by cytochrome oxidase, an enzyme which is known to be inhibited by chlorpromazine *in vitro* (Bernsohn, Namajuska and Boshes, 1956; Dawkins, Judah, and Rees, 1959).

Costa and Himwich (1958) found a lowered 5-hydroxytryptophan decarboxylase activity in rabbit brain after convulsive doses of insulin, although the concentration of brain 5-hydroxytryptamine was raised or unaltered. Their results suggest that insulin, like chlorpromazine, depresses turnover of cerebral 5-hydroxytryptamine.

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GANGLION-BLOCKING PROPERTIES OF ATROPINE-LIKE DRUGS

BY

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The ganglion-blocking properties of atropine, atropine methyl nitrate, oxyphenonium, poldine, methantheline and propantheline have been examined and compared with those of hexamethonium, mecamylamine, pentolinium and tetraethylammonium. The ganglion-blocking activity was assessed by stimulating the preganglionic nerve to the superior cervical ganglion and recording directly the percentage depression in the postganglionic action potentials. In this way any ambiguity due to the peripheral actions of the drugs was removed. As a group the atropine-like compounds are not markedly less potent than the ganglion-blocking agents, but their action is relatively transient. This work suggests that the ganglion-blocking action of atropine-like substances plays no part in their therapeutic effects.

Atropine-like drugs are frequently used in clinical practice for their peripheral anti-acetylcholine effects. The actions of atropine-like drugs at peripheral sites have been fully investigated, but their actions at autonomic ganglia have been examined in less detail [Marrazzi (1939), Dutta (1949), Konzett and Rothlin (1949), Ambache (1949), Cahen and Tvede (1952), and Paton (1954)]. We therefore thought it important to find out whether these drugs produce significant ganglion-block in doses which antagonize the peripheral actions of acetylcholine.

The measurement of ganglion-blocking action has usually been carried out on the cat nictitating membrane preparation, but there are several objections to this, particularly for atropine-like drugs which have a considerable peripheral action. For example, Cervoni, West, and Fink (1956) found that atropine directly depressed the smooth muscle of the nictitating membrane. This was confirmed by Thompson (1958) using the isolated nictitating membrane preparation. Even direct perfusion of the ganglion with the drug does not eliminate this error, since up to 50% may not reach the ganglion, being diverted possibly by arterio-venous anastomoses to peripheral sites (Paton, 1954).

In this investigation we have avoided complications due to peripheral actions of the drugs by measuring directly the postganglionic nerve potentials evoked by stimulating the preganglionic nerve to the superior cervical ganglion. We have studied the ganglion-blocking activity of several atropine-like drugs and some true ganglion-blocking agents.

METHODS

Stimulation and recording of postganglionic action potentials were similar to the method described by Eccles (1935).

Anaesthesia was induced in cats with ether, followed by intravenous chloralose/urethane solution (1% and 5% respectively) at approximately 5 ml./kg. The cervical sympathetic nerve and the postganglionic nerve from the superior cervical ganglion were dissected out. Care was taken to leave the blood supply to the ganglion intact. The cat was then transferred to a metal screening cage, stimulating and recording electrodes were placed in position and the exposed tissues were covered with liquid paraffin.

A rectangular pulse of approximately 0.1 msec. duration, 2 to 10 V. and 2 pulses/sec. frequency was applied to the preganglionic nerve. The recording electrodes on the postganglionic nerve led to a condenser-coupled amplifier and oscilloscope with recording camera.

Drugs were given intravenously into the femoral vein. The action potentials were photographed immediately before and at 1 min. after the injection, the effect of the compounds being maximal at this time. The action of the drug was expressed as the percentage reduction of the spike amplitude as measured from the photographic record.

Four to eight observations were made with each drug. The response was plotted against the dose on a log scale and a regression line was fitted by eye to the points obtained. The ED50 was read directly from the graph.

The duration of action of the atropine-like drugs was determined by injecting a dose equal to the ED50 and photographing the action potential before administration and at 1, 2, 4, and 8 min. after administration. The action potentials following hexamethonium were measured at 5 min. intervals until recovery. The percentage depression of spike amplitude was plotted against time, and from the graph the time taken from maximum depression to half recovery was estimated. Two experiments were carried out with each drug.

The following salts of the various drugs were used: pentolinium tartrate, hexamethonium bromide, mecamylamine hydrochloride, atropine sulphate, tetraethylammonium bromide, methantheline bromide, propantheline bromide, oxyphenonium bromide, and poldine methosulphate (Nacton).

The doses given below are expressed in terms of these salts.

Results

Fig. 1 illustrates the action potentials before and after the administration of 0.2 mg./kg. of hexamethonium and 4 mg./kg. of atropine. This dose of atropine reduced the spike potential by approximately 80%, and hexamethonium by about 35%.

Fig. 2 shows the dose-response lines obtained for most of the drugs investigated. The slopes of the lines would appear to be all of the same order.

The doses of atropine-like drugs and ganglion-blocking agents giving a 50% reduction in spike amplitude, as determined from the dose-response lines, are given in Table I.

It will be noted that the atropine-like drugs, as a group, are somewhat less potent than the true ganglion-blocking agents, but the difference is not marked. In the case of atropine methyl nitrate this drug is even more potent than mecamylamine and hexamethonium. However, the two groups of drugs differ considerably in their duration of action. The atropine-like drugs have a short effect of approximately 2 to 6 min., while the duration of action of the true ganglion-blocking drugs administered at the ED50 level is upwards of 30 min.

A comparison of the duration of action of atropine-like drugs with hexamethonium was made and the results are given in Table II. As the time of complete recovery was rather variable, and as the initial slope of the recovery curve was more constant, the "mean half recovery time" as defined above was worked out.

Table I

RELATIVE GANGLION-BLOCKING POTENCY OF ATROPINE-LIKE DRUGS AND TRUE GANGLION-BLOCKING AGENTS WITH RESPECT TO HEXAMETHONIUM

Drug	ED50 (mg./kg.)	Equipotent Molar Ratio (Hexamethonium = 1.0)
Pentolinium	0·07	6·55
Mecamylamine ..	0·23	0·733
Hexamethonium ..	0·31	1·0
Atropine methyl nitrate	0·14	2·22
Propantheline	0·70	0·545
Methantheline	0·70	0·510
Tetraethylammonium ..	0·80	0·226
Atropine	2·4	0·249
Oxyphenonium ..	2·4	0·154
Poldine (Nacton) ..	3·8	0·097

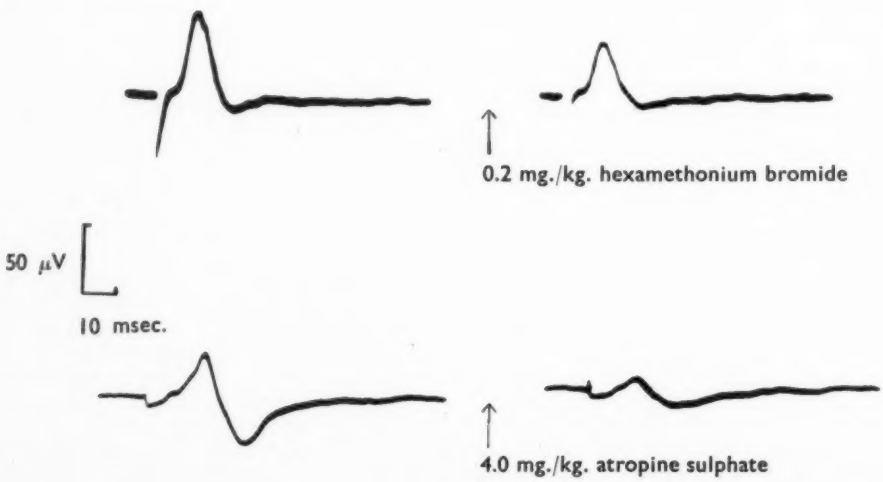

Fig. 1.—Postganglionic action potentials. Top records: before and after the intravenous injection of 0.2 mg./kg. of hexamethonium. Bottom records: before and after 4 mg./kg. of atropine.

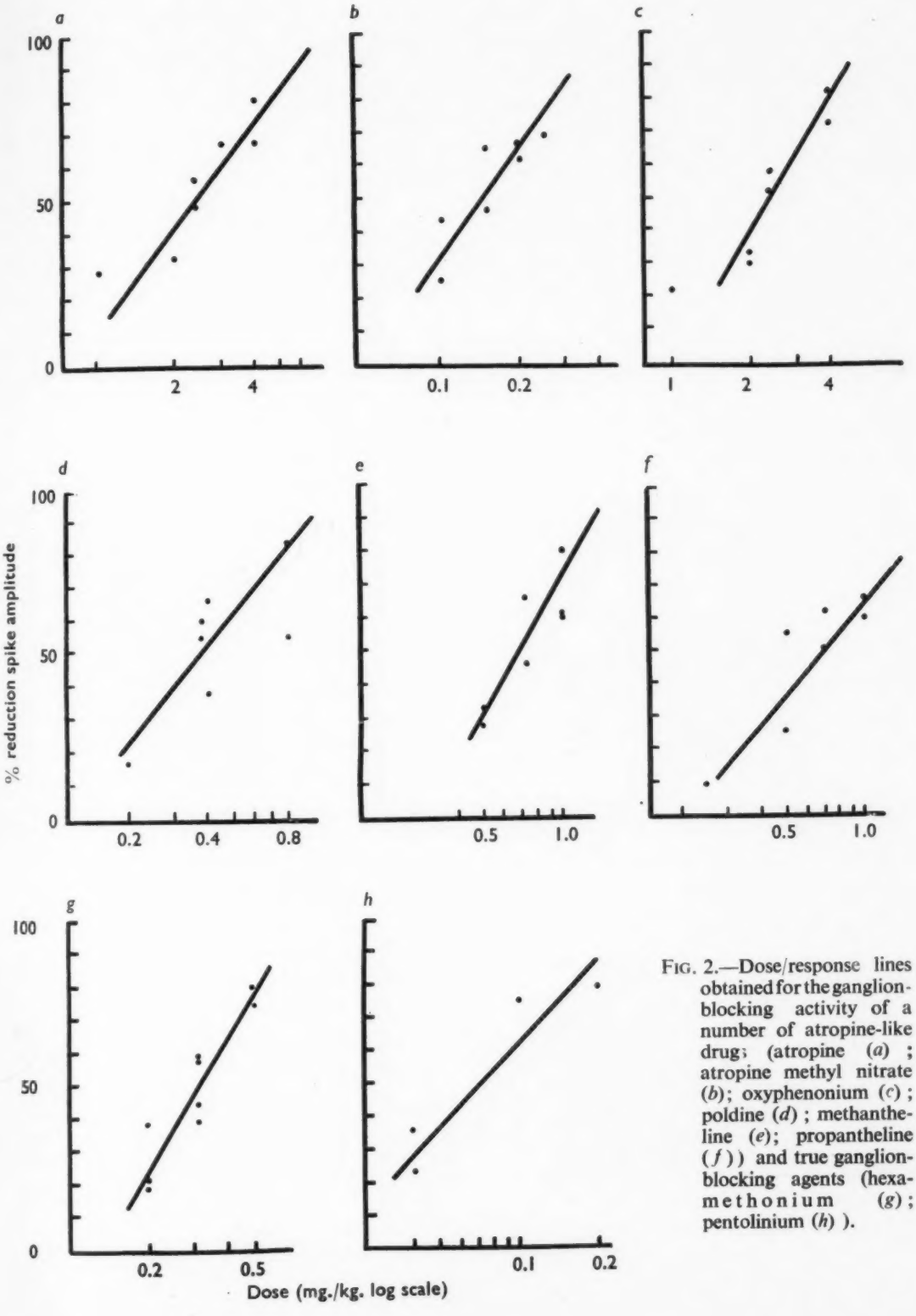

FIG. 2.—Dose/response lines obtained for the ganglion-blocking activity of a number of atropine-like drugs (atropine (*a*); atropine methyl nitrate (*b*); oxyphenonium (*c*); poldine (*d*); methantheline (*e*); propantheline (*f*)) and true ganglion-blocking agents (hexamethonium (*g*); pentolinium (*h*)).

TABLE II

TIME TAKEN FOR THE POSTGANGLIONIC ACTION POTENTIAL TO RECOVER BY 50% AFTER THE ADMINISTRATION OF THE ED50 OF SOME ATROPINE-LIKE DRUGS AND HEXAMETHONIUM

Drug	Mean Half Recovery Time (Min.)
Oxyphenonium	5·5
Atropine	4·2
Atropine methyl nitrate	4·7
Poldine	5·8
Propantheline	3·3
Methantheline	2·3
Hexamethonium	31·0

DISCUSSION

Atropine and various anti-acetylcholine drugs have been shown by a direct method to block transmission through the superior cervical ganglion.

Fig. 3*a* shows the relation between toxic, ganglion-blocking, and peripheral anti-acetylcholine activity of the atropine-like drugs, and Fig. 3*b* shows the relationship between the toxic and ganglion-blocking doses for the true ganglion-blocking drugs. The toxic dose is the intravenous LD50 for mice, and the figure for anti-acetylcholine activity is the dose required to obtain 50% inhibition of the vasodepressor response to intravenous acetylcholine in the cat (Acred, Atkins, Bainbridge, Brown, Quinton, and Turner, 1957). The ganglion-blocking dose is the ED50 value as given in Table I.

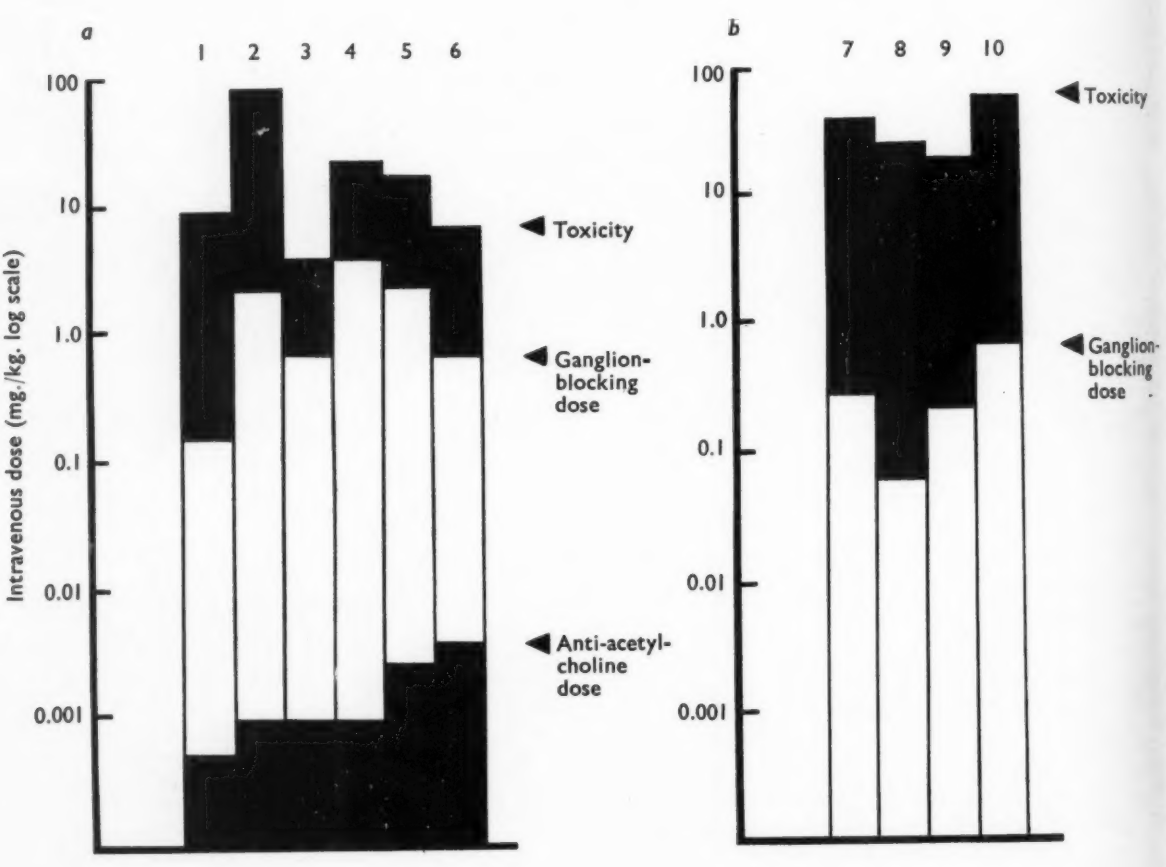

FIG. 3.—Diagram showing (*a*) relationship between toxicity, ganglionic and peripheral activities of atropine-like compounds (1, atropine methyl nitrate; 2, atropine; 3, propantheline; 4, poldine; 5, oxyphenonium; 6, methantheline); and (*b*) toxic and ganglionic activity of ganglion-blocking agents (7, hexamethonium; 8, pentolinium; 9, mecamylamine; 10, tetraethylammonium bromide).

In the case of the ganglion-blocking agents the ratio between toxic and ganglion-blocking doses is large, ranging from 400 for pentolinium to 90 for mecamylamine and tetraethylammonium, whereas in the atropine-like group the ratio varies from 6 for propantheline to 60 for atropine methyl nitrate. ED50 values for the ganglion-blocking activity for the two groups actually overlap, atropine methyl nitrate being remarkably potent whereas tetraethylammonium is less potent than methantheline and propantheline. The ratio between ganglionic and peripheral ED50 values in the atropine-like group is of the order of 1,000, and so it is most improbable that ganglion-block contributes to their therapeutic actions.

No simple relation exists between ganglionic and peripheral potencies in the atropine group. Quaternization of the nitrogen in atropine leads to an increase of toxicity and peripheral anti-acetylcholine activity and to a proportionately much greater ganglion-blocking potency. The presence of a quaternary nitrogen atom is, however, not essential to a high ganglion-blocking potency (for example, mecamylamine and pempidine).

The effect of anti-acetylcholine drugs at ganglia could be due to depolarization or competition with acetylcholine. Paton and Perry (1953) have shown that acetylcholine, nicotine, and tetramethylammonium block ganglia by depolarization, all causing an initial stimulation of the ganglion cells. Other known ganglion-blocking agents neither depolarize nor cause an initial stimulation and are assumed to act competitively. We have no evidence that the atropine-like drugs cause an initial stimulation of the superior cervical ganglion, although we did produce an initial stimulation of the intestine *in vivo* after intravenous administration (Acred *et al.*, 1957). It is possible that this is a nicotine-like effect. On the other hand, Konzett and Rothlin (1949), perfusing the superior cervical ganglion and recording from the nictitating membrane, overcame the block produced by atropine with higher concentrations of acetylcholine, suggesting a competitive mode of action.

The ganglion-blocking action of atropine can be attributed partly to its local anaesthetic activity (Paton, 1954). However, most of the compounds we have investigated are quaternary esters and have no local anaesthetic properties (Acred *et al.*, 1957; Barlow, 1955). Further, while atropine has about half the local anaesthetic potency of procaine the latter has a much smaller ganglion-blocking action than atropine both by intravenous and close intra-arterial injection to the ganglion (see also Dutta, 1949). Hence the available evidence as to mode of action suggests a competitive block such as probably occurs at peripheral sites, although other modes of action are possible (Paton, 1954).

Although the ganglionic action of atropine could hardly become manifest in clinical use it could explain the observation that in anaesthetized cats intravenous doses greater than 1 mg./kg. often cause a transient fall of blood pressure.

We can conclude that the ganglion-blocking action of atropine-like drugs does not add significantly to their peripheral actions. In the first instance, the dose required to produce a ganglion-block is so great that the degree of side-effects alone would prohibit their use. In the second instance, the duration of block is so short that in no way could it be said to influence the therapeutic action. Finally, the precise mechanism of the block has not as yet been elucidated.

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PHARMACOLOGICALLY ACTIVE PEPTIDES IN THE BLOOD AND URINE OF ANIMALS INFECTED WITH *BABESIA RODHAINI* AND OTHER PATHOGENIC ORGANISMS

BY

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From the Wellcome Laboratories of Tropical Medicine, London

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The blood and urine of mice and rats infected with *Babesia rodhaini* contain substances which stimulate the isolated guinea-pig ileum and rat duodenum. The amount of active material excreted increases as the infection increases. The active substances are stable to boiling with hydrochloric acid but not with alkali; they pass through a cellophane membrane and are soluble in hot ethanol. They are destroyed rapidly by papain and less rapidly by chymotrypsin, but are unaffected by trypsin or pepsin. Their action on smooth muscle is not affected by atropine, eserine, antihistamines, iproniazid, bretylium or by lysergic acid diethylamide. The active substances are probably peptides and there is evidence that the urine contains a mixture of peptides, some of which relax and some of which contract the rat duodenum. Similar active peptides appear in the urine of mice infected with *Plasmodium berghei*, *Trypanosoma rhodesiense*, *Streptococcus pyogenes* and Rift Valley fever virus.

Maegraith, Gilles and Devakul (1957) made a study of the pathological processes in puppies and dogs infected with *Babesia canis*. They showed that in severe and fatal cases the clinical progress bore "little direct relation to the prevailing degree of parasitic infection of the erythrocytes," and that the plasma often became lytic for both parasitized and normal erythrocytes. After severe lysis, the erythrocyte count, haemoglobin and packed cell volume often remained within normal limits, although the animal passed into a state resembling oligaemic shock. An injection of noradrenaline caused immediate, though temporary, recovery from this condition; a similar dramatic effect was reported by Maegraith, Devakul and Leithead (1956) in monkeys infected with *Plasmodium knowlesi*.

Maegraith *et al.* (1957) concluded that the pathological processes are non-specific and are common to other acute medical states, and that "the initiating factors may be physiologically active soluble substances of relatively simple nature derived from the parasite or as a consequence of host-parasite reaction or arising from tissue damage."

Severe or fatal shock also occurs in cattle infected with piroplasms, and may be brought about by treatment with effective drugs (Stephan and Esquibel, 1929). A similar phenomenon is sometimes observed in mice infected with *B. rodhaini* (Beveridge, 1953). It has been suggested that the destruction of parasites by the drug liberates substances which are toxic to the host. The liberation of "toxins" from protozoal parasites has often been suggested to explain physiological disturbances in the host, but pharmacological studies of the nature of these "toxins" have been few.

The present paper describes a preliminary study of the active substances present in the blood and urine of animals infected with *B. rodhaini* and other pathogenic organisms. Pharmacologically active peptides are shown to be present.

METHODS

Infections

Babesia rodhaini.—The strain used was the rat-adapted strain described by Beveridge (1953). Mice (20 g.) or rats (50 g.) were inoculated intraperitoneally with 0.1 ml. of infected rat blood containing 100 to 600m. parasitized red cells. The strain has increased in virulence since 1953 and now kills all mice in about 5 days. Rats did not die; maximal parasitaemia was reached in 5 to 6 days and then declined. In most experiments the parasites were counted daily in stained blood smears.

Plasmodium berghei.—The strain was obtained from the London School of Hygiene in 1949 and maintained in mice by blood passage. Mice were

infected by the intraperitoneal injection of 0.1 ml. of infected blood containing about 500 million parasitized red cells. The infection reached its maximum in the blood in 8 days; most of the mice died 6 to 8 days after inoculation.

Trypanosoma rhodesiense.—The strain (C) was isolated in Entebbe in 1939 and maintained in mice by blood passage. Mice were infected by the intraperitoneal injection of 0.5 ml. of diluted infected blood containing about 500,000 trypanosomes; the mice died 4 days after inoculation.

Streptococcus pyogenes.—A culture of a freshly isolated strain was obtained from University College Hospital. Mice were inoculated intraperitoneally with 0.05 ml. of a 24-hr. subculture on Loeffler's serum agar. The mice died 4 days after inoculation.

Rift Valley Fever.—The strain was maintained by passage of infected liver. Mice were inoculated intraperitoneally with infected liver suspension; they died 4 days after inoculation.

Intravascular Haemolysis without Infection

Groups of 10 mice were injected intravenously with preparations which caused or simulated intravascular haemolysis and gave rise to haemoglobinuria. The preparations included haemoglobin solution, lysed mouse red cells, washed rabbit red cells, saponin and phenylhydrazine.

Collection of Urine

Urine was collected in a glass metabolism cage from groups of 10 to 50 mice. Faeces were separated with a terylene net screen, or by means of a polythene funnel with a curved spout similar to the glass funnel described by Brittain (1959). Urine was usually collected in a bottle containing 0.05 ml. of N HCl, and was neutralized to *p*H 7.2 before testing. In some experiments the collecting vessel was immersed in alcohol and solid carbon dioxide so that the urine was frozen as soon as it reached the bottle. Mice were fed daily for 3 hr. outside the cage; the rest of the time between inoculation and death was spent in the cage; the urine collected was not contaminated with food. Water was unrestricted at feeding time; in the metabolism cage it was provided in a bottle or 1 ml. was given daily into the stomach of each mouse to provide a good urine flow. Collected specimens of urine were stored at $-15°$. In some experiments, urine was collected with a pipette directly from the mouse after gentle pressure on the abdomen. In some experiments the urine was passed through alumina columns (Savory and Moore, chromatographic grade) before testing.

Preparation of Extracts

Blood and urine samples were collected in siliconed apparatus. Extracts of urine, blood and tissues were prepared by Code's modification (1937) of Barsoum and Gaddum's (1935) method for the assay of histamine.

Urine was dialysed for 24 hr. in a cellophane sac against an equal volume of water and the fluids on both sides of the membrane tested. Urine was also dialysed against running water for 24 hr.

Extracts of blood and urine were also prepared with hot ethanol. Two volumes of boiling absolute ethanol were added to the sample, and boiling continued for 10 min. The liquid was centrifuged and filtered and the filtrate evaporated to dryness under reduced pressure at less than 50°. The residue was dissolved in a small amount of glacial acetic acid and precipitated by the addition of 9 vols. of anaesthetic ether. The precipitate was washed with ether, dried and dissolved in de Jalon solution.

A crude separation of peptides was made by the method of Gaddum and Horton (1959). 25 ml. of urine of mice infected with *Babesia* was passed through a column (10×1 cm.) of amberlite IRC 50 resin adjusted to *p*H 6.0. The liquid which passed through the column was reserved. The column was washed with phosphate buffer at *p*H 6.0 and then adjusted to *p*H 9.0 by the addition of the calculated amount of N NaOH. Elution was then carried out with M phosphate buffer at *p*H 9.0 and serial fractions of 2.5 ml. of eluate were collected. Part of each fraction was used for test and part was evaporated to dryness, extracted with hot ethanol, and the extract evaporated and dissolved in de Jalon solution for test. All samples and extracts were adjusted to *p*H 7.2 before test.

Isolated Organs

Guinea-pig ileum was suspended in a bath of Tyrode solution at 37°. Rat duodenum was prepared by the method of Horton (1959) and suspended in a 3 ml. bath of de Jalon solution at 31°. Atropine (10^{-6}) was used for both preparations to depress spontaneous movements. Atropine, mepyramine, triprolidine, eserine, iproniazid, bretylium and lysergic acid diethylamide were tested as antagonists, usually at a concentration of 10^{-4} in the bath.

Enzymes

Urine specimens and hot ethanol extracts were incubated for 1 to 24 hr. at 37° with proteolytic enzymes and the effect on the response of the rat duodenum tested. Controls were set up containing all reagents except the enzyme and containing normal mouse urine instead of infected mouse urine.

Chymotrypsin.—0.5 mg. was added to 0.25 ml. of sample in 0.1 M $NaHCO_3$ buffer.

Trypsin.—10 mg. was added to 0.25 ml. of sample in 0.1 M $NaHCO_3$ buffer.

Pepsin.—5 mg. was added to 0.5 ml. of sample in N/10 HCl.

Papain.—10 mg. was added to 0.5 ml. of sample in buffer at *p*H 6.0 containing 2 mg. of cysteine.

Samples of urine were also heated with N/10 NaOH in the boiling water bath for 10 min. and neutralized. Their activities were compared with unheated samples containing equivalent amounts of salt.

Haemoglobin was determined in urine by the acid haematin method.

Potassium was determined in blood and urine in the flame photometer.

Results

It seemed possible that liberation of histamine might be responsible for shock in infected animals, and accordingly an attempt was made to determine the histamine content of urine, blood, skin, nose and feet of rats and mice during the course of infection with *B. rodhaini*. It was soon apparent that the urine and tissues of infected animals contained substances which interfered with the assay of histamine. An example is shown in Fig. 1; an extract of blood which contained no detectable histamine caused a slow contraction of the guinea-pig ileum which persisted on washing out. Sensitivity to subsequent standard doses of histamine was greatly increased.

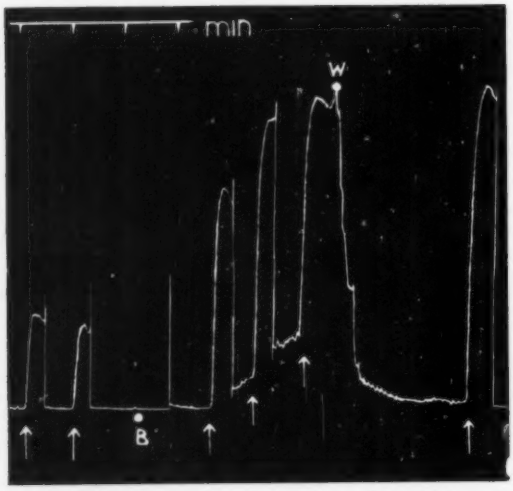

FIG. 1.—The effect of an extract of blood from mice infected with *Babesia* on the response of the guinea-pig ileum to histamine. Tyrode solution containing atropine 10^{-6}; 10 ml. bath. At arrows: 30 ng. histamine. At B: 1 ml. extract of *Babesia* blood (Code process). Wash periods: 30 sec., except at W, which was for 2.5 min.

Fig. 2 shows that hot ethanol extracts of infected blood caused larger contractions of guinea-pig ileum than an extract of normal blood.

Large amounts of active material were found in the urine of infected mice and rats. Fig. 3 shows the contractions of the guinea-pig ileum caused by a series of daily specimens of urine collected from mice during the 5 days of the infection. Fig. 4 shows that the appearance of

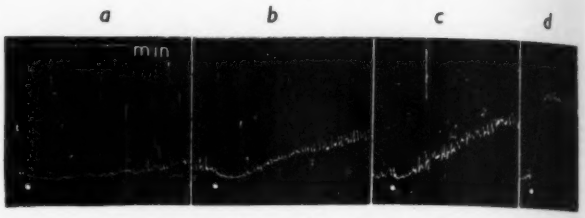

FIG. 2.—The effect of hot ethanol extracts of the blood of mice infected with *Babesia* on the isolated guinea-pig ileum. Tyrode solution containing atropine 10^{-6}; 10 ml. bath. 1 ml. ethanol extracts of mouse blood at (*a*) normal blood; (*b*) blood containing 1% of parasitized erythrocytes; (*c*) blood containing 17% of parasitized erythrocytes. At (*d*), 500 ng. histamine.

active substances in the urine preceded the rise of parasitaemia and haemoglobinuria. This occurred both in mice which died 5 days after inoculation and in rats which recovered from the infection spontaneously.

In many experiments with guinea-pig ileum, a slight relaxation or inhibition of spontaneous movements preceded the contraction caused by *Babesia* urine. The initial relaxation was much more marked when the urine was tested on the isolated rat duodenum (Horton, 1959). Relaxation was usually slight at the beginning of the infection and increased from day to day; on the day before death the relaxation was much greater than the subsequent contraction (Fig. 5).

The responses of different specimens of rat duodenum were variable. The typical reaction to *Babesia* urine was a relaxation followed by a contraction, but with some preparations relaxation predominated and, with others, contraction. The response depended partly on the load on the lever; lightly loaded preparations were more

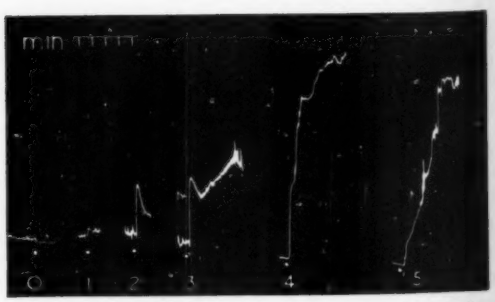

FIG. 3.—The effect of urine from mice infected with *Babesia* on the isolated guinea-pig ileum. Tyrode solution containing atropine 10^{-6}; 3 ml. bath. At 0, 0.1 ml. urine collected before infection; at 1, 2, 3, 4 and 5, 0.1 ml. urine collected on 1st, 2nd, 3rd, 4th and 5th days of the infection. The mice died after the 5th day.

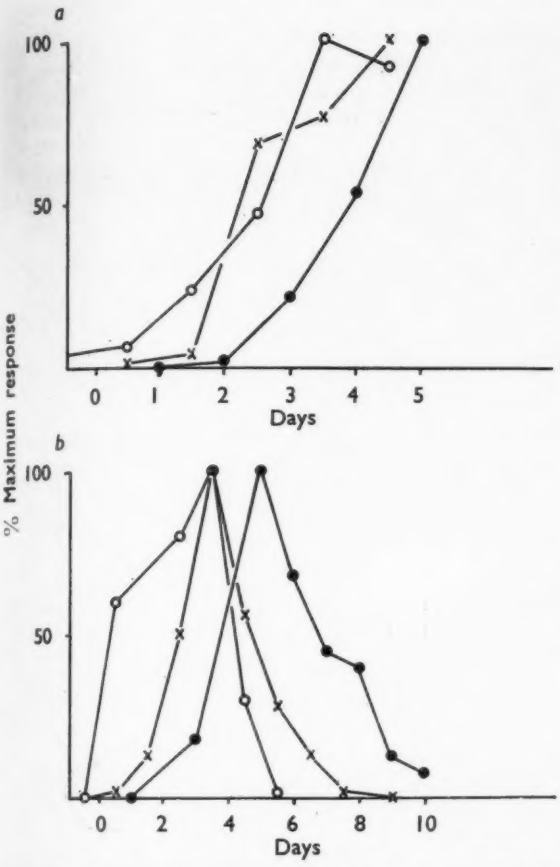

FIG. 4.—The progress of *Babesia* infections in mice (*a*) and rats (*b*) as measured by parasitaemia (●——●), haemoglobinuria (X——X) and the activity of the urine on the isolated guinea-pig ileum (O——O). Measurements are plotted as percentages of the maximum. The maximum percentage of parasitized erythrocytes in mice was 94% (5th day) and in rats 42% (6th day). The maximum degree of haemoglobinuria in mice was 21.2 mg./ml. and in rats 7.5 mg./ml. The effect on the ileum was measured as the height of the contraction produced in experiments similar to that illustrated in Fig. 3.

likely to relax well and more heavily loaded preparations were more likely to contract. A load of 0.5 g. as recommended by Horton (1959) was found to be the optimum for showing both responses.

Normal mouse or rat urine sometimes gave a small contraction and sometimes a small relaxation; the effect was never as large as that of *Babesia* urine. Urine collected from mice inoculated intraperitoneally with normal mouse blood showed no increase in active substances.

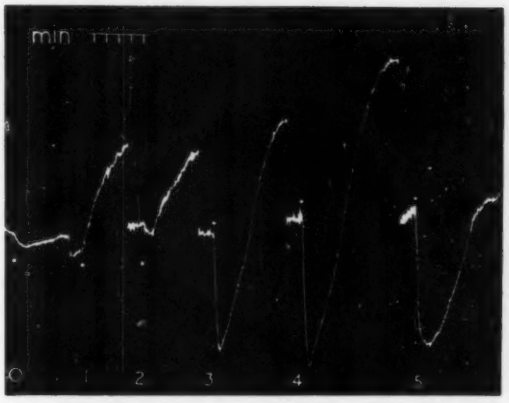

FIG. 5.—The effect of urine from mice infected with *Babesia* on the isolated rat duodenum. De Jalon solution with atropine 10^{-6}; 3 ml. bath. The samples of urine were the same as those in Fig. 3; the dose was 0.05 ml.

Bradykinin always gave a relaxation which seldom recovered as far as the original base-line until the bath was washed out.

The variable effect of *Babesia* mouse urine on the rat duodenum and the day-to-day qualitative changes which took place as the infection progressed suggested that there was probably more than one active substance in the urine. When a specimen of mouse urine which caused contraction of the rat duodenum was passed through an Amberlite IRC 50 column at *p*H 6.0 the filtrate gave a pure relaxation. When the column was eluted at *p*H 9.0 the first fraction collected caused relaxation; this fraction would be expected to contain kinins (Gaddum and Horton, 1959). The second fraction gave a contraction followed by a relaxation and subsequent fractions gave pure contractions (Fig. 6). The maximal response was given by the fifth sample collected. Hot ethanol extracts of the fractions gave responses of the rat duodenum almost identical with those of the fractions from which they were prepared. The activity of the fractions was unchanged by passage through alumina columns; the active substances passed readily through a cellophane membrane.

When urine from infected mice, or the material extracted from it by hot ethanol, was incubated with trypsin or pepsin, the activity was not destroyed in 24 hr. Papain destroyed all activity in 1 hr. Incubation with chymotrypsin for 1 hr. increased activity; incubation for 24 hr. destroyed almost all activity (Fig. 7). Boiling with alkali reduced, but did not abolish, activity. Active substances were present in urine collected

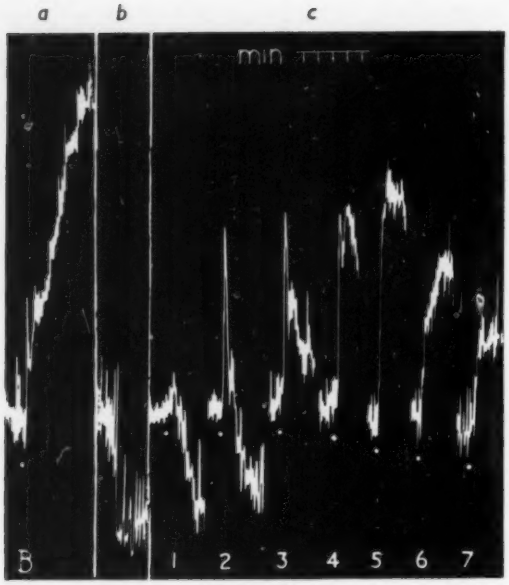

FIG. 6.—The separation of active fractions from the urine of mice infected with *Babesia*. Rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath. (*a*): at B, untreated *Babesia* urine; (*b*): fluid which passed through Amberlite IRC 50 column at *p*H 6.0; (*c*): at 1–7, successive fractions collected from the column washed with buffer at *p*H 9.0. All doses 0.03 ml.

directly from the mouse by pressure on the abdomen and were stable in the refrigerator at −15°. Specimens stored in a refrigerator at 4° showed partial conversion of relaxing to contracting activity.

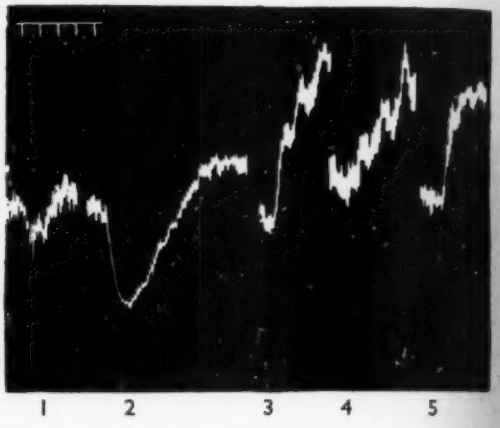

FIG. 8.—Active substances in the urine of mice in which haemoglobin has been released intravascularly. Isolated rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath. Doses 0.02 ml. Time in min. 1, normal mouse urine; 2, *Babesia* mouse urine; 3, 4 and 5, urine from mice injected intravenously with lysed mouse blood, saponin, and rabbit erythrocytes respectively.

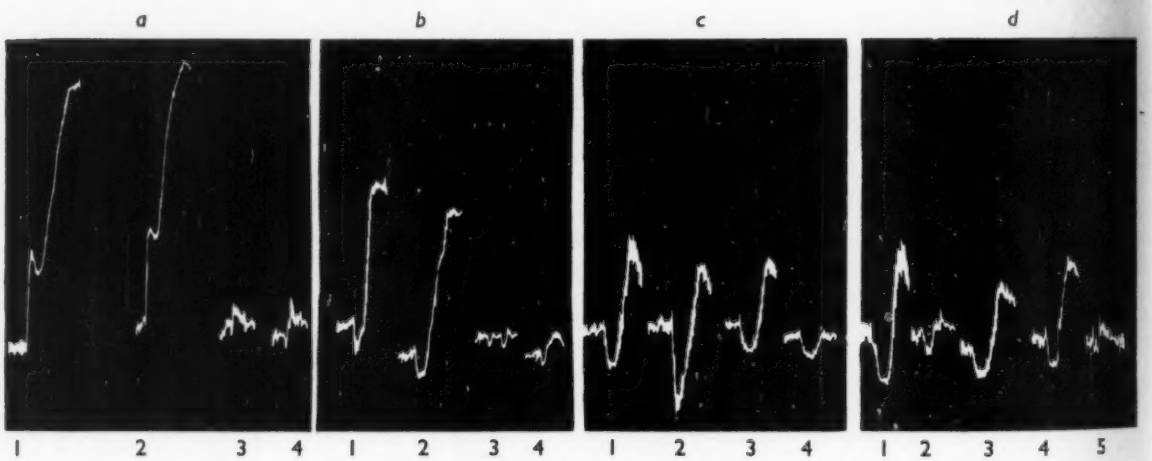

FIG. 7.—Effect of enzymes on urine of mice infected with *Babesia*, assayed on isolated rat duodenum. De Jalon solution with atropine 10^{-6}; 3 ml. bath. All doses 0.03 ml. (*a*) Pepsin: 1, *Babesia* urine incubated 24 hr. alone; 2, *Babesia* urine incubated 24 hr. with pepsin; 3, normal urine incubated 24 hr. alone; 4, normal urine incubated 24 hr. with pepsin. (*b*) Trypsin: 1, *Babesia* urine incubated 24 hr. with trypsin; 2, *Babesia* urine incubated 24 hr. alone; 3, normal urine incubated 24 hr. alone; 4, normal urine incubated 24 hr. with trypsin. (*c*) Chymotrypsin: 1, *Babesia* urine incubated 1 hr. alone; 2, *Babesia* urine incubated 1 hr. with chymotrypsin; 3, *Babesia* urine incubated 24 hr. alone; 4, *Bab sia* urine incubated 24 hr. with chymotrypsin. (*d*) Papain: 1, *Babesia* urine, fresh; 2, *Babesia* urine, incubated 1 hr. with papain; 3, *Babesia* urine, incubated 1 hr. alone; 4, *Babesia* urine, incubated 24 hr. alone; 5, *Babesia* urine, incubated 24 hr. with papain.

Experiments in which haemoglobin, lysed washed mouse erythrocytes, rabbit erythrocytes or haemolytic drugs were injected intravenously into mice showed that in all instances pharmacologically active substances were excreted in the urine. The quantities were rather less than would have been expected in *Babesia* urine showing the same degree of haemoglobinuria, and the relaxing component was apparently absent (Fig. 8). Haemoglobin, in larger amounts than those present in *Babesia* urine, when given alone or with normal mouse urine had no effect on the isolated guinea-pig ileum or rat duodenum. Urine mixed with fresh mouse blood had activity which in some instances was similar to that of *Babesia* urine.

Atropine, mepyramine, tripolidine, eserine, iproniazid, bretylium and lysergic acid diethylamide at a concentration of 10^{-4} in the bath had no effect on the relaxation or contraction of rat duodenum caused by *Babesia* urine or extracts of urine (Fig. 9). Higher concentrations of lysergic acid diethylamide had no effect on the relaxation but sometimes reduced the subsequent contraction of the rat duodenum.

Mice infected with *Plasmodium berghei*, *Trypanosoma rhodesiense*, *Streptococcus pyogenes* and Rift Valley fever virus all excreted substances in the urine which caused contraction or relaxation of the rat duodenum (Fig. 10). In acute fatal infections, the amount of relaxant material increased greatly towards the end.

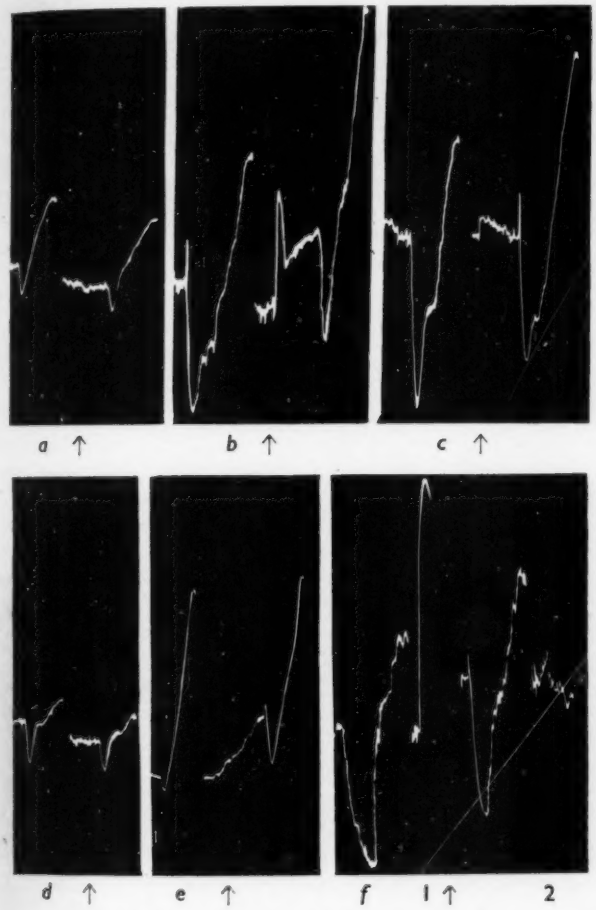

FIG. 9.—The lack of effect of various drugs on the response of the isolated rat duodenum to urine of mice infected with *Babesia*. De Jalon solution with atropine 10^{-6}; 3 ml. bath. The effect of a dose of 0.02 ml. of urine was recorded and the bath washed out. The drug was added (↑) to give a concentration of 10^{-4} in the bath and a second dose of 0.02 ml. urine then added without washing out. (*a*) Atropine, (*b*) mepyramine, (*c*) iproniazid, (*d*) eserine, (*e*) bretylium, (*f*) lysergic acid diethylamide; 5-hydroxytryptamine was given at 1 (0.005 ml. 10^{-4} solution) and at 2 (0.01 ml. 10^{-4} solution); the bath was washed out between the second dose of urine and the second dose of 5-hydroxytryptamine.

DISCUSSION

The cause of death of animals infected with protozoa is often obscure. Post-mortem examinations frequently show that the organs, although not normal, exhibit little evidence of severe impairment of function. *Babesia rodhaini* infections in mice are often heavy, and death may often be accounted for by the severity of the anaemia. However, the death of puppies infected with *B. canis*, and of mice treated with a non-toxic dose of an effective drug, cannot be explained in this way. The results of the present investigation show that the blood and urine of infected animals contain substances which are active on smooth muscle, some of which are probably peptides. They are stable to boiling with hydrochloric acid but not with alkali, they are extractable with hot ethanol, they pass readily through cellophane, they are destroyed by papain and chymotrypsin but not by trypsin or pepsin. Their action on smooth muscle is not affected by antihistamines, atropine, eserine, or by lysergic acid diethylamide in doses which abolish the action of 5-hydroxytryptamine. Identification of the active constituents will depend upon the separation of relatively pure fractions from the mixture of substances which has been shown to be present. Until this is done, quantitative parallel assays are of doubtful value.

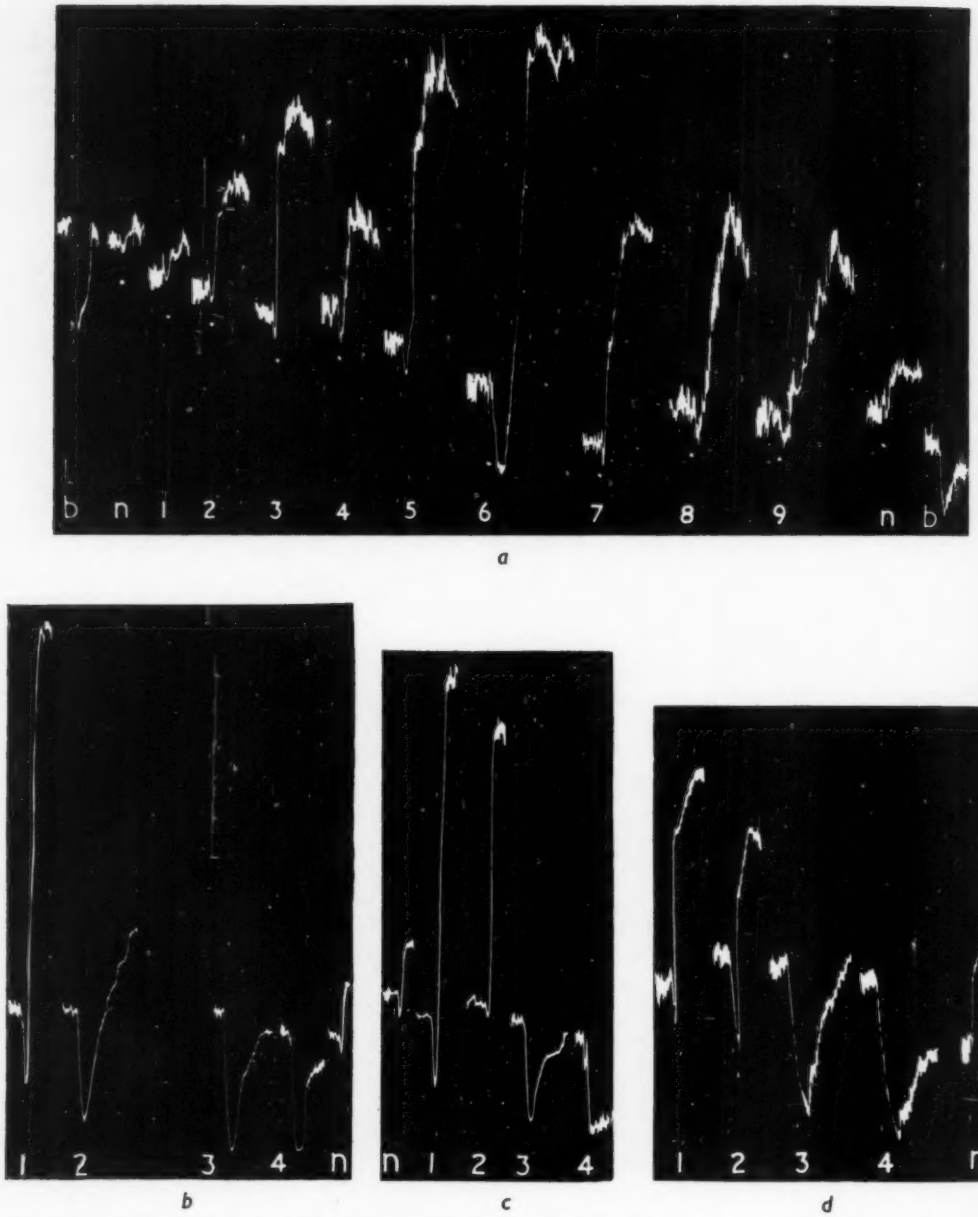

FIG. 10.—Active substances in the urine of mice infected with various organisms. Isolated rat duodenum in de Jalon solution with atropine 10^{-6}; 3 ml. bath; doses of urine 0.02 ml. b= bradykinin (10^{-6} in bath); n=normal mouse urine collected in metabolism cage; 1–9=urine samples collected daily until death. (*a*) *Plasmodium berghei*; (*b*) *Streptococcus pyogenes*; (*c*) Rift Valley fever; (*d*) *Trypanosoma rhodesiense*.

It is of interest to speculate upon the origin of the active substances which appear in the blood and urine of infected animals. *Babesia* and malaria parasites live within the red cells and destroy haemoglobin; they must possess powerful proteolytic enzymes. At schizogony, the erythrocyte bursts and the merozoites, together with their metabolic residues, are released into the plasma. It is possible that protein residues may be released from the broken erythro-

cytes and also that free enzymes derived from the parasite may attack plasma proteins and release peptides. In addition, the presence of broken cells and parasites in the plasma may start the chain of reactions which give rise to the release of kinins from plasma globulins. The experiments in which intravascular haemolysis was induced in mice by the injection of drugs or of lysed or incompatible red cells show that haemolysis is followed by the appearance of active substances in the urine.

Beraldo (1952) described substance U, a peptide in dog urine which was formed by the mixture of urine with traces of blood. He suggested that the release of substance U might explain some of the symptoms of pathological conditions in which blood and urine come into contact (Beraldo, 1955). The present experiments are clearly related to Beraldo's work and extend it into the field of the pathology of infectious diseases.

Pharmacologically active peptides occur not only in the urine of animals infected with haemolytic organisms, but also in trypanosome and virus infections. It is likely that the sensitization of guinea-pig ileum to histamine demonstrated in Fig. 1 is connected with the histamine sensitization which occurs in pertussis infections of mice (Parfentjev and Goodline, 1948). Matsui and Kuwajima (1959) have recently shown that the histamine sensitizing factor is "a toxin which has nothing to do with preventive antigens."

It seems possible that active peptides are liberated from the proteins of the host in all serious illnesses and that their pharmacological effects may be associated with the symptoms and signs of infectious disease.

We are grateful to Dr. E. W. Horton for helpful advice and a gift of bradykinin, to Miss E. Beveridge for help with the *Babesia* strains, and to Miss S. Hook for technical assistance.

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METABOLISM AND EXCRETION OF DI(*p*-AMINOPHENYL) SULPHOXIDE IN DIFFERENT ANIMAL SPECIES

BY

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Rabbits, rats and guinea-pigs were treated with di(*p*-aminophenyl) sulphoxide and their urines examined by an analytical method which permits the simultaneous determination of this compound and of dapsone [di(*p*-aminophenyl) sulphone] which is a possible product of metabolic oxidation. The method gives for each drug the total of free compound plus acid-labile conjugates. All three species excreted unchanged drug together with dapsone. With rats and guinea-pigs about 33% of the excretion is dapsone, but with rabbits only 6 to 12%. The rate of combined excretion is much greater in rabbits than in the other two species. These results are discussed in relation to the significance of di(*p*-aminophenyl) sulphoxide as a drug in the treatment of leprosy.

Di(*p*-aminophenyl) sulphoxide has received favourable preliminary reports in clinical trials against leprosy (Buu-Hoï, Khuyen, and Xuong, 1955; Davey, Kissaun, and Moneta, 1957; Laviron, Lauret, Kerbastard, and Jardin, 1957), and there has been a suggestion that its action might be in some ways different from that of dapsone, di(*p*-aminophenyl) sulphone. Various workers have shown the sulphoxide to be almost without action *in vitro* against micro-organisms including pneumococci, haemolytic streptococci, and *Mycobacterium tuberculosis* H37Rv, in contrast to the considerable activity of dapsone in these species. However, the sulphoxide has definite activity *in vivo* against these same organisms in the mouse and the guinea-pig. These findings have led to the suggestion that the sulphoxide is activated by conversion to the sulphone in the animal body (Jensen and Schmith, 1943; Youmans, Feldman, and Doub, 1946; Wagner and Kimmig, 1946).

In order to evaluate the specific usefulness of the sulphoxide in leprosy it is desirable to know the extent of its conversion to dapsone in the human body. The metabolic fate of sulphoxides varies with different compounds and in different species. A number of instances are known where thioethers are oxidized to sulphoxides and are excreted in this form rather than as sulphones, for example, in the metabolism of phenothiazine (Whitten, Filmer, and Clare, 1947), chlorpromazine (Salzman and Brodie, 1956), and a pyrazolidinedione containing an alkylthio group (Burns, Yü, Ritterband, Perel, Gutman, and Brodie, 1957); the oxidation of di(*p*-aminophenyl) sulphide by a guinea-pig liver microsome preparation is said to go to the sulphoxide but no further (Gillette, Kamm, and Brodie, 1959). On the other hand, sulphides can be oxidized *in vivo* to the sulphone, for example, *p*-methylthioaniline (Rose and Spinks, 1948), and the catabolism of ethanethiol probably follows this route after methylation (Snow, 1957; Lowe, 1960). These oxidations probably involve formation of the sulphoxide as an intermediate step.

A method has been developed for the determination of di(*p*-aminophenyl) sulphoxide and dapsone in the presence of each other. It has been used to investigate metabolism in three animal species and is now available for investigation of metabolism in man.

Bushby and Woiwod (1956) showed that dapsone is largely excreted as the mono-*N*-glucuronoside. Our method therefore includes a preliminary acid treatment to hydrolyse such conjugates, and the results show the sum of free plus acid-labile compounds.

METHODS

Various methods of distinguishing between di(*p*-aminophenyl) sulphoxide and dapsone were considered. The sulphoxide gives a colour reaction with concentrated sulphuric acid (100° for 10 min.) whilst the sulphone does not. However, the intensity of the colour is far too low for convenience ($\varepsilon = 670$ at 520 mμ). A method has now been

developed for separating the sulphoxide from the sulphone by differential extraction from an organic solvent with acids of differing strength; this enables a diazotization method similar to that of Francis and Spinks (1950) to be applied to both components.

Procedure.—Urine samples (5 ml.) are acidified with N HCl (2.5 ml.); they are left for 1 hr. at 20 to 25° to allow for hydrolysis of acid-labile conjugates. After neutralization with N NaOH (2.5 ml.) the samples are diluted to contain 3 to 40 μg./ml. of either sulphoxide or sulphone. For estimation 1 ml. is mixed with 3 ml. 0.2 M phosphate buffer *p*H 7.0. Blood samples (3 ml.) are mixed with 0.8 M phosphate buffer *p*H 7.0 (1 ml.).

For the first extraction, 4 ml. of buffered urine or blood, prepared as above, is shaken with isobutyl methyl ketone (20 ml.). Both sulphoxide and sulphone pass quantitatively into the organic phase.

For the second extraction, a 15 ml. aliquot of the separated ketone layer is extracted with 0.1 N HCl (4 ml.); 88.9% of the sulphoxide and 2.3% of the sulphone pass from the ketone solution into the acid layer. Of the acid layer 2 ml. is mixed with 0.1% $NaNO_2$ solution (1 ml.), and 5 min. later 1% sulphatoethyl-*m*-toluidine solution (0.5 ml.) is added. After 20 min., ethanol (3 ml.) is added and the optical density is read at 520 mμ. A standard curve, which should be rectilinear, is prepared from readings on solutions containing 2 to 20 μg. di(*p*-aminophenyl) sulphoxide in 2 ml. 0.1 N HCl diazotized and coupled as above. The apparent sulphoxide content of the aliquot of 0.1 N HCl from the second extraction is read from the standard curve. Let this value be x μg.

For the third extraction, a 10 ml. aliquot of the ketone layer remaining after the extraction with 0.1 N HCl is further extracted with 2N HCl (4 ml.). The small residue of sulphoxide is quantitatively extracted into the stronger acid, together with 93.8% of the remaining sulphone. Of the acid layer, 3 ml. is taken and mixed with 0.1% $NaNO_2$ solution (0.5 ml.). After 5 min. 5% ammonium sulphamate solution (1 ml.) is added, followed 5 min. later by 0.2% *N*-1-naphthylethylenediamine solution (0.5 ml.). After a further 30 min. the solution is diluted to 6 ml. and the optical density read at 535 mμ. A standard curve (rectilinear) is constructed from readings on solutions containing 2 to 20 μg. of dapsone in 2N HCl (2 ml.) diazotized and coupled as above. The apparent dapsone content of the aliquot of 2N HCl from the third extraction is read from the standard curve. Let this value be y μg.

The amounts of sulphoxide and sulphone in the buffered solution taken for the first extraction are given by the equations:

di(*p*-aminophenyl) sulphoxide content (μg.) ... ... $=3.01x-0.075y$

dapsone content (μg.) ... ... $=2.92y-0.36x$

(The derivation of these equations is shown below.) From these figures the concentrations present in the original blood or urine may be calculated, taking into account the dilutions involved in making the buffered solution for extraction.

General Precautions.—The isobutyl methyl ketone is presaturated with phosphate buffer, and the 0.1 N and 2N HCl solutions with isobutyl methyl ketone, in order to avoid volume changes during the extraction. All extractions are carried out by shaking the solutions for 1 min. in stoppered tubes and centrifuging the mixtures to separate the layers cleanly. Nitrite and *N*-1-naphthylethylenediamine solutions are freshly prepared each day. Stock solutions of the pure drugs contained 1 mg./ml. in dilute acid; the sulphone may be kept several weeks, but the sulphoxide only for three days. Diluted solutions (10 μg./ml.) are prepared as required. Colours formed after coupling fade in strong daylight, and the tubes are kept in the dark except during the actual measurement. Solutions are thoroughly mixed after each addition of reagent. This is especially important after ethanol additions, where faulty mixing can easily occur.

Analysis of the Method.—Coupling of diazotized dapsone with sulphatoethyl-*m*-toluidine does not occur satisfactorily in 2N HCl, and for the third extraction a coupling component which requires this strength of acid was substituted. Di(*p*-aminophenyl) sulphoxide and dapsone at equal concentrations diazotize and couple with sulphatoethyl-*m*-toluidine to produce colours of almost equal intensity at 520 mμ. The same is true of the colours produced with *N*-1-naphthylethylenediamine measured at 535 mμ. Thus no appreciable error arises when measurements are made on mixtures containing both amino compounds.

The formula for calculating the results was derived from a series of experiments in which standard solutions of di(*p*-aminophenyl) sulphoxide and dapsone were extracted under conditions identical to those used in the procedure described above. Extraction into 0.1 N and 2N HCl was determined directly using standard solutions of the sulphoxide and sulphone in isobutyl methyl ketone (saturated with buffer). Extraction into isobutyl methyl ketone was determined indirectly: standard solutions of sulphoxide and sulphone in phosphate buffer *p*H 7.0 were extracted into isobutyl methyl ketone and an aliquot of the ketone layer was extracted with HCl (0.1 N for the sulphoxide, 2N for the sulphone). The efficiency of the first extraction step was calculated from knowledge of the efficiency of the overall extraction and that of the extractions with acid. Each extraction was carried out several times. Mean results are given in Table I.

Using these figures, equations may be derived as follows:

Let the prepared sample of blood or urine (4 ml.) contain A μg. of sulphoxide and B μg. of sulphone. This sample is quantitatively extracted into isobutyl methyl ketone (20 ml.). A 15 ml. aliquot thus contains 0.75 (A+B): it is extracted with 0.1 N HCl (4 ml.), and the measurement (x) is made on a 2 ml.

aliquot of the acid. From the determined extraction efficiencies

$$x = 0.5 \times 0.75\ (0.889A + 0.023B)$$
$$\therefore\ x = 0.334A + 0.0086B \quad \ldots\ldots\ldots\ldots\ldots\ldots (1)$$

After this extraction a 10 ml. aliquot of the ketone layer contains $0.5\ [\ (-0.889)\ A + (1 - 0.023)\ B]$; it is extracted with 2N HCl (4 ml.), and the measurement (y) is made on a 3 ml. aliquot of the acid. From the determined extraction efficiencies

$$y = 0.75 \times 0.5\ (0.111A + 0.938 \times 0.977B)$$
$$\therefore\ y = 0.0415A + 0.344B \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

Solving the simultaneous equations (1) and (2) gives the expressions for A and B quoted in the section on procedure.

The complete method was tested by determination of known mixtures of the two components in water and by determinations on blood and urine to which known amounts had been added. The results (Table II) show that some sets of figures have a small bias, but without any consistent trend. The standard deviation was quite similar for each set and could be taken as ±5% for the method as a whole. This is satisfactory for practical requirements.

Animal Experiments.—The animals were fed on normal laboratory diets and food was withdrawn 18 hr. before the administration of the drug. The compound was dosed in aqueous suspension by means of a stomach tube. The urine from the animals in metabolism cages was collected in a container chilled in ice, acidified, and extracted as soon as practicable after excretion. For rats and guinea-pigs the numbers of animals were sufficient to ensure a representative excretion of urine over the periods involved. With rabbits excretion was promoted by giving 15 ml. water at the time of treatment, but even so it was inconveniently erratic. This was partly overcome by repeating the dosing and collection from each individual rabbit after a rest period of three days. The urines from the two collections were combined as far as possible, as shown in Table IV. Where there was an excretion in only one of the pair of experiments over a given period figures are shown in brackets. These figures are based on the assump-

TABLE I

EXTRACTION OF DI(*p*-AMINOPHENYL) SULPHOXIDE AND DAPSONE

	1st Extraction. From *p*H 7·0 Buffer into Isobutyl Methyl Ketone		2nd Extraction. From Isobutyl Methyl Ketone into 0·1 N HCl		3rd Extraction. From Isobutyl Methyl Ketone into 2N HCl	
	Sulphoxide	Sulphone	Sulphoxide	Sulphone	Sulphoxide	Sulphone
Phosphate buffer *p*H 7·0 (ml.)	4	4	—	—	—	—
Isobutyl methyl ketone (ml.)	20	20	15	15	10	10
Hydrochloric acid	—	—	4 ml. 0·1 N	4 ml. 0·1 N	4 ml. 2 N	4 ml. 2 N
Wt. of compound taken (μg.)	10–20	10–20	10–20	125–750	10–20	10–20
No. of determinations ..	9	9	9	12	9	9
Mean % extraction	101·6	99·9	88·9	2·31	101·8	93·8
Standard error on mean ..	0·6	0·8	0·6	0·01	1·6	0·5

TABLE II

RESULTS OF ESTIMATIONS ON SYNTHETIC MIXTURES OF DI(*p*-AMINOPHENYL) SULPHOXIDE AND DAPSONE

	In Water		In Urine		In Blood	
Number of determinations ..	12		10		6	
Component	Sulphoxide	Sulphone	Sulphoxide	Sulphone	Sulphoxide	Sulphone
Range of amounts of components in mixtures (μg.) ..	5·2–48	7·4–37·2	1·6–21·6	15·4–46·2	10·5–101·5	20·8–131
Mean % error on result ..	−2·4	2·7	3·4	−3·3	−5·5	−0·6
Standard deviation of % error	5·0	4·4	3·9	6·2	2·1	4·5

TABLE III

URINARY EXCRETION FROM GUINEA-PIGS AND RATS DOSED WITH DI(*p*-AMINOPHENYL) SULPHOXIDE

Guinea-pigs (wt. 250 g.) each received 2·5 mg. di(*p*-aminophenyl) sulphoxide orally. Rats (wt. 300 g.) each received 3 mg. of the drug orally. Figures for both species refer to 2 separate groups of 6 animals. Cumulative % figures for dapsone are based on the sulphoxide equivalent.

Species	Time after Dose hr.	Sulphoxide Excretion				Dapsone Excretion			
		mg.		Cumulative % of Dose		mg.		Cumulative % of Dose	
Guinea-pig	0–3	0·38	0·59	2·5	4·0	0·19	0·21	1·0	1·5
	3–6	1·07	0·58	9·5	8·0	0·68	0·27	5·5	3·0
	6–12	0·40	0·46	12·5	11·0	0·26	0·30	7·0	5·0
	12–24	0·35	0·26	14·5	12·5	0·18	0·10	8·0	5·5
Rat	0–3	1·72	1·61	9·5	9·0	0·61	0·68	3·0	3·5
	3–6	2·12	1·36	21·5	16·5	1·11	0·74	9·0	7·5
	6–12	1·39	1·06	29·0	22·5	0·88	0·64	13·5	10·5
	12–24	0·59	0·51	32·5	25·0	0·37	0·34	15·5	12·5

TABLE IV

URINARY EXCRETION FROM RABBITS DOSED WITH DI(*p*-AMINOPHENYL) SULPHOXIDE

Each animal received 10 mg. per kg. of di(*p*-aminophenyl) sulphoxide orally on two separate occasions. Cumulative % figures for dapsone are based on the sulphoxide equivalent. Values in parentheses refer to a single experiment only.

Collection Period (hr. after Dose)	Expt. No.	Sulphoxide Excretion		Dapsone Excretion	
		mg.	Cumulative % of Dose Excreted	mg.	Cumulative % of Dose Excreted
Rabbit 1. *Dose:* 30 *mg. in each experiment*					
0–2	1+2	20·9	35	0·0	0
2–5	2	3·68	(48)	0·33	(1·0)
2–12	1	2·62	47	0·32	2·0
5–12	2	1·03		0·21	
12–24	1+2	1·15	49	0·28	3·1
Rabbit 2. *Dose:* 20 *mg. in each experiment*					
0–2	3+4	16·1	40	1·69	4·2
2–6	3+4	2·99	48	0·50	5·5
6–12	3	0·49	(50)	0·15	(6·2)
6–24	4	1·77	51·5	0·46	7·3
12–24	3	0·26		0·14	

tion that the rate of excretion was identical in the two experiments with the same animal. Results from two rabbits are presented in Table IV as typical.

RESULTS

The analytical method has been used to investigate the oxidation of di(*p*-aminophenyl) sulphoxide to dapsone in guinea-pigs, rats, and rabbits. The dose of drug (10 mg./kg.) was comparable, on the basis of the surface area of the body, with therapeutic doses used in man. At this dose the blood levels were very low, and only urinary excretion has been studied in detail. Results are given in Tables III and IV.

The total excretion of sulphoxide and sulphone is much less with the guinea-pig than with the rat, but the pattern of excretion is similar in these species. In both, the excretion at 3 hr. is about one third, and at 6 hr. two thirds, of the total 24 hr. excretion. There is considerable oxidation of the compound in the body and, of the detectable excretion products, about one third is dapsone and two thirds sulphoxide. The excretion in rabbits is quite different. Elimination is much more rapid; about 75% of the 24 hr. excretion occurs in the first 2 hr. Also the conversion to dapsone is much less. The fraction of diazotizable material excreted in the sulphone form was 6, 12, 10, and 6% in four different experiments.

DISCUSSION

There are clearly differences in the metabolism of di(*p*-aminophenyl) sulphoxide in different species, though some conversion to dapsone may perhaps be expected in all cases. The smaller extent of oxidation in the rabbit is probably the result of the more rapid excretion of the sulphoxide.

Since this work was carried out a paper has appeared (Jardin, 1958) reporting the chromatographic investigation of urine from patients receiving di(*p*-aminophenyl) sulphoxide. The results show qualitatively that the unchanged drug is probably the principal excretion product; dapsone is found in somewhat smaller amounts along with some relatively minor metabolites. Jardin expresses his opinion that the amount of dapsone excreted is not sufficient to account for the antileprotic activity of the sulphoxide. This point, however, can only be convincingly proved when quantitative information on the blood levels and urinary excretion of the two compounds is obtained. The method described in this paper should enable this to be done.

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DEPLETION OF PITUITARY CORTICOTROPHIN BY RESERPINE AND BY A NITROGEN MUSTARD

BY

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A single intraperitoneal injection of reserpine (2.5 mg./kg.) into rats produced a fall in the corticotrophin concentration of the pituitary to 30% of the resting value; recovery was not far from complete at 40 hr. A single injection of a nitrogen mustard caused an even greater loss of pituitary corticotrophin; 24 hr. after the injection the concentration was 10% of the resting value. There is no reason to assume that the effect of reserpine is due to an interference with storage of ACTH in the tissue and is not simply due to the fact that the drug acts as a stressing agent. The shape of the curve representing the fall in pituitary ACTH during the early phases of a sudden stress may be very similar to that of the fall in adrenal ascorbic acid produced by the released ACTH. This suggests that, under these circumstances, resynthesis is slow and the diminishing stores of ACTH in the pituitary reflect mainly the release of the hormone.

It is well known that reserpine depletes tissues of their stores of amines, such as the catecholamines and 5-hydroxytryptamine. Does reserpine also deplete stores of peptidic substances, such as the pituitary hormones ? To attempt to answer this question, the content of corticotrophin in the pituitary of the rat was estimated at intervals after the administration of a large dose of reserpine. The effects were compared with those of the severe stress of an injection of a nitrogen mustard. In all animals the adrenal ascorbic acid was also measured.

METHODS

Drug Administration

Young adult female rats, weighing about 200 g. and obtained from a single colony, were used throughout. They were fed on cubes and water and kept at 23°. Rats injected with the same solution were kept together in a cage. Injections were made without previous "training." At different intervals after the injection pairs of rats were removed from each cage, rapidly decapitated, and estimations of pituitary ACTH and adrenal ascorbic acid carried out. The following injections were given:

Reserpine.—A solution containing 2.5 mg. of reserpine per ml. ("Serpasil" ampoules, Ciba Ltd.) was injected intraperitoneally in a dose of 1 ml. per kg.

Vehicle.—The vehicle for the reserpine, supplied by Ciba Ltd., was injected intraperitoneally in volumes of 1 ml./kg.

Saline.—A 0.9% solution of sodium chloride was injected intraperitoneally in volumes of 1 ml./kg.

Nitrogen Mustard.—10 mg. of di(2-chloroethyl) methylamine hydrochloride ("Mustine," Boots) was taken up in 6 ml. of water. 1 ml. (=1.7 mg.) was injected subcutaneously into each rat.

Assay of Corticotrophin

Rats.—Young adult male rats, ranging in weight from 180 to 200 g., were used in the bioassay of corticotrophin. They were obtained from 3 different sources: the animals from one source showed much greater variability of response than the others; the best index of precision (λ) was obtained with rats from Messrs. A. J. Tuck, Rayleigh, Essex.

Extraction of Pituitary Gland.—The glands of the injected animals were carefully dissected from the base of the skull. Usually the posterior lobe separated easily. The anterior lobe was quickly weighed on a torsion balance and was placed into a thick-walled test tube (7 mm. inside diameter × 75 mm.), containing 0.5 ml. glacial acetic acid and a few grains of finely ground washed sand. The tissue was crushed and ground thoroughly with a glass rod, enlarged at the end to fit the bottom of the tube. The rod was drained on the side of the test tube, the tube was covered with a square of parafilm and was heated in a sand bath at 70 to 80° for 30 min. The tubes were centrifuged for 5 min. and aliquots of the supernatan.

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fluid dried overnight in a vacuum desiccator over NaOH. The residues were stored in the freezer (−17°). In one experiment, posterior lobes were treated in a similar way, but the tissue of three rats was pooled.

Bioassay.—The *in vitro* bioassay procedure of Saffran and Schally (1955) was used with some modifications. Four, instead of 8, rats were used in each assay, permitting one person to carry out 2 assays per day unaided, with some loss in precision (average λ close to 0.2). Ethylene chloride was used instead of methylene chloride for the extraction of the steroids. This substitution was made necessary by the lack of a cooling system on the lamp housing of the Unicam spectrophotometer. Ethylene chloride is not transparent to ultra-violet light at wavelengths much below 240 mμ, so that it was not possible to examine the shape of the absorption curve of the extract by taking a reading at 230 mμ. Incubation of the adrenal tissue was carried out in 5 ml. vials in a shaking incubator instead of in a conventional Warburg apparatus. The vials were gassed by blowing out the air with a strong stream of 95% oxygen containing 5% carbon dioxide, and quickly stoppering with a rubber stopper while the stream of gas played on the mouth of the vial.

The residues of 0.1 to 0.25 ml. of the original pituitary extracts were taken up in 0.5 ml. of 0.25% aqueous acetic acid, and 0.1 ml. portions of these solutions were used as the U_2 doses in the 4-point bioassay, while 0.1 ml. of 1 in 4 dilutions in 0.25% acetic acid were used as U_1. A solution of a commercial preparation of corticotrophin ("Cortrophin," Organon) in 0.25% acetic acid was used as a laboratory standard, with S_2 = 20 mU. and S_1 = 5 mU. in volumes of 0.1 ml. added per incubation vial.

Determination of Adrenal Ascorbic Acid

Extraction of the Tissue.—While the pituitary gland was being removed from the head, the adrenals were excised from the body, freed of adhering fat, weighed in pairs on a torsion balance, and ground in a small test tube (10 × 75 mm.) in 0.3 ml. of 4% trichloroacetic acid with finely ground sand and a glass rod enlarged at one end. The rod was washed down with the trichloroacetic acid solution to a total volume of 1.2 ml. The tubes were then covered with parafilm and were stored in the freezer.

Measurement of Ascorbic Acid.—A modification of the method of Roe and Kuether (1943) was used. The adrenal extracts were thawed and about 50 mg. of acid-washed charcoal was added to each tube. The tubes were again covered with the parafilm and were shaken vigorously for 15 sec. A pellet of cotton-wool, fitting the tube snugly, was slowly pushed down the tube with a glass rod. As the cotton-wool passed through the extract, the particles of charcoal and precipitated protein were trapped by the fibres, and a clear solution passed through to the top of the pellet. An aliquot (usually 0.03 or 0.04 ml.) of the clear solution was removed from each tube to a series of fresh similar tubes.

To these aliquots were added one drop of the thiourea reagent and 0.3 ml. of the dinitrophenylhydrazine reagent. The tubes were covered with parafilm and were placed in boiling water for exactly 15 min. (Guillemin, Fortier, and Lipscomb, 1959) and then cooled in ice water. To each tube was added 1 ml. of ice-cold 85% (v/v) sulphuric acid. The tubes were covered with parafilm and inverted to mix the contents. The colours were read in microcells in the Unicam spectrophotometer at 520 mμ. A calibration curve was contructed with ascorbic acid solutions in 4% trichloroacetic acid taken through the whole procedure.

Results

Saline.—Four hours after the injection of 0.9% saline, the content of corticotrophin in the pituitary had fallen to 50% of the value at zero time, while at 8 hr. the amount had returned nearly to the starting level (Fig. 1a). At 40 hr. the values were again lower.

The ascorbic acid in the adrenals was not significantly lowered in this experiment.

Vehicle.—The administration of the reserpine vehicle produced signs of discomfort in the animals. The corticotrophin decreased to about 45% at 8 hr., then returned to about 60% at 18 hr. and remained there (Fig. 1b).

The adrenal ascorbic acid also fell to a minimum at 4 and 8 hr., with subsequent recovery. There is no doubt that the vehicle acted as a stress but that recovery was rapid.

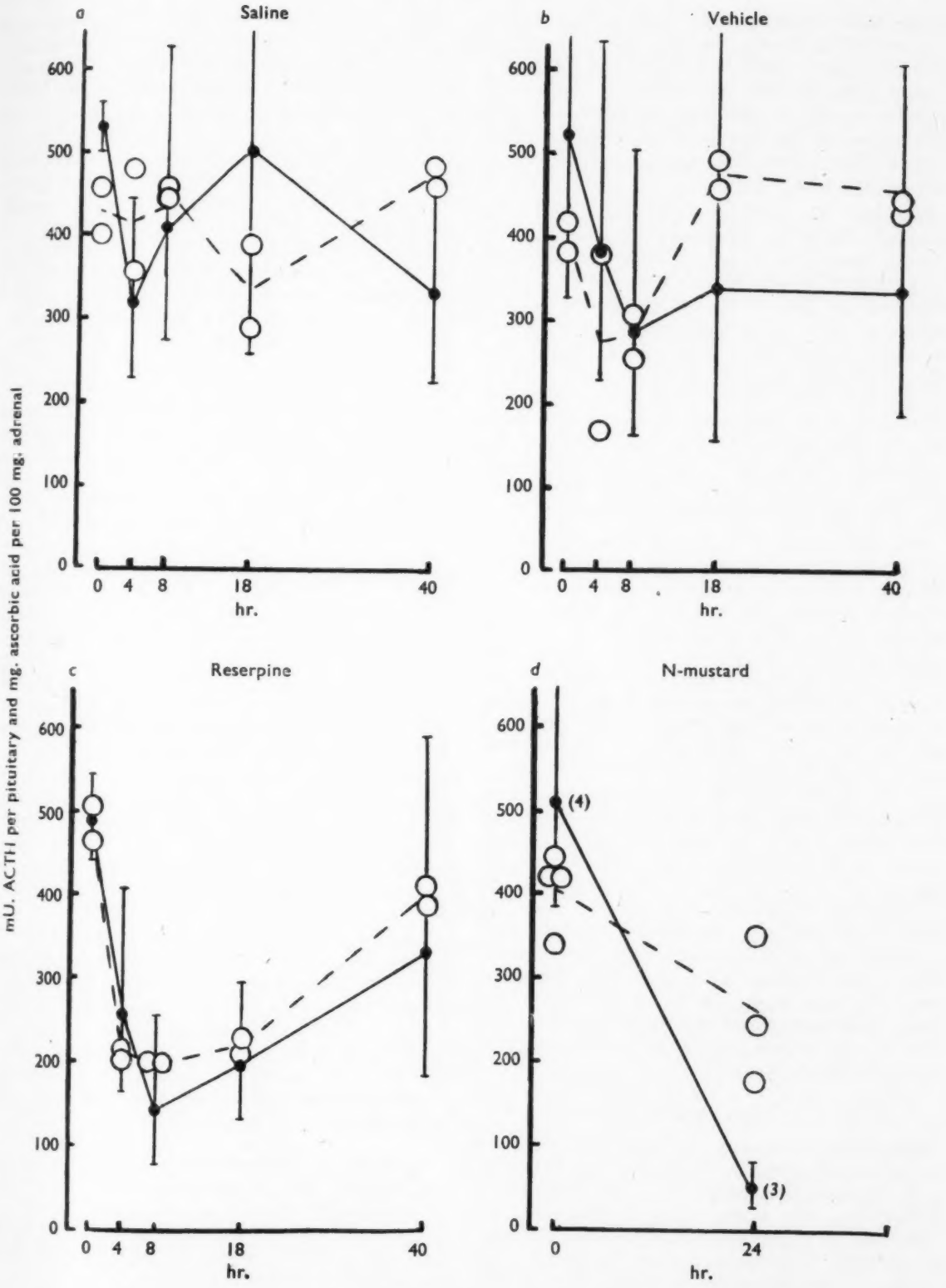
Reserpine.—After reserpine, the corticotrophin decreased to a minimum of 30% at 8 hr., thereafter slowly returning to about 75% at 40 hr. (Fig. 1c).

The adrenal ascorbic acid fell to a minimum of 40% at 4 hr. and 8 hr., then gradually returned to 80% at 40 hr., the time course of the changes closely resembling that of the changes in corticotrophin concentrations.

The losses in ACTH were of a degree not previously encountered after the injection of drugs, but of the magnitude reported (Fortier, 1959;

FIG. 1.—a, b and c: ●——● mU. ACTH per anterior lobe, each dot being the mean for a pair of rats, the vertical strokes representing the confidence limits of the estimation (P=0·95). – – – – mean adrenal ascorbic acid concentrations for the same pairs of rats. **O** individual ascorbic acid values.

FIG. 1.—d: ●——● mU. ACTH per anterior lobe, means and confidence limits (P=0·95) of 4 controls and 3 experimental animals. – – – – mean adrenal ascorbic acid concentrations for the same rats. **O** individual ascorbic acid values.


a
Saline
b
Vehicle
c
Reserpine
d
N-mustard
mU. ACTH per pituitary and mg. ascorbic acid per 100 mg. adrenal
0
100
200
300
400
500
600
0 4 8 18 40
hr.
(4)
(3)
0 24

Fortier and DeGroot, 1959) after adrenalectomy (fall to 31%) or after removal of one and enucleation of the other adrenal (fall to 23%). In order to help in the interpretation of the results, it was thought desirable to find out what response would follow a single injection of a severely toxic drug. Thus the next experiment was carried out with a nitrogen mustard.

Nitrogen Mustard. — At 8 hr. after the administration of the drug there were few signs of toxicity, and the five animals were therefore left for a total of 24 hr. By that time two of the rats had died, and the survivors exhibited oedema and weakness. The corticotrophin in the pituitary of the survivors had fallen to 10% (Fig. 1d), and the adrenal ascorbic acid was at 70% of the starting value. The values for ascorbic acid at zero time were lower in this group of rats than in the groups used for the other treatments, and therefore the fall to 70% (or 260 mg./100 g. adrenal) represented a concentration of ascorbic acid which approached the minimum figures observed in stress.

One experiment of longer duration was carried out, in which the action of reserpine was compared with that of an unspecific stressing agent—the reserpine vehicle—and in which the behaviour of the stores of ACTH in the posterior lobe of the pituitary was also examined. The posterior lobe contains an appreciable amount of ACTH which has been shown to be released in certain forms of stress (Mialhe-Voloss, 1956, 1958 ; Rochefort and Saffran, 1957). Reserpine (2.5 mg./kg.) was injected intraperitoneally on 4 consecutive days, and the ACTH estimated separately in anterior and posterior lobe 24 hr. after the last injection. Rats injected with the same volume of vehicle served as controls. There was no difference in the concentration of ACTH in pituitaries of rats injected with reserpine and injected with the vehicle, and this was true for the ACTH in the anterior lobe as well as for that in the posterior lobe. The absolute amount in both lobes was about one-half of the figures for unstressed rats. After prolonged treatment, therefore, there was also no sign of specific interference of reserpine with storage of ACTH.

Discussion

Reserpine is known to interfere with the transport and storing mechanism of catecholamines and 5-hydroxytryptamine, but not with that of histamine. The reason for this difference is unknown, as is the exact nature of the processes transporting and storing amines in certain cells against a concentration gradient. The present work was done with the purpose of finding out whether the storage of pituitary hormones, for example, ACTH, was interfered with by reserpine. The results showed a large drop of ACTH in pituitaries after a single injection of reserpine. The interpretation was complicated by the necessity of assessing the extent to which reserpine would lower ACTH concentration simply because it acts as a stressing agent. The conclusion reached was that the release of ACTH after reserpine could be explained entirely on the basis of its stressing action ; one reason for this conclusion was the observation that a very toxic substance, a nitrogen mustard, caused an ever greater loss of ACTH ; the second was the short duration of the effect : practically normal concentrations were found 40 hr. after a single injection, whereas reserpine-depleted stores of 5-hydroxytryptamine take many days to fill up again. Since formation of 5-hydroxytryptamine is a very fast process (Brodie, Spector, Kuntzman, and Shore, 1958), the delay can only be accounted for by a prolonged action of the reserpine ; such action should affect all substances, the storage of which is interfered with, for the same length of time. Lastly, repeated injections of reserpine lowered the stores of ACTH to the same extent as repeated injections of the solvent, and this finding applied equally to the stores in the anterior as to those in the posterior lobe, where the ACTH might be held by a different chemical process. At neither site was there any indication that reserpine produced effects not also obtained by other noxious agents.

At least one other polypeptide is known, on which reserpine has no depleting action, namely substance P. Studies of its concentration in brain (Paasonen and Vogt, 1956) have shown that it remains unchanged after the injection of doses of reserpine which cause a severe fall in the content of 5-hydroxytryptamine.

The simultaneous estimation of the concentrations of adrenal ascorbic acid and pituitary corticotrophin shows that, in short-term experiments, the general trend of the changes is often similar. Thus, during periods of a sudden increase in demand on ACTH, release of hormone appears not to be balanced by resynthesis, so that the resulting fall in pituitary stores can be used as an index of increased secretion.

Reserpine (" Serpasil ") and its vehicle were kindly supplied by Ciba Laboratories Ltd., and ACTH (" Cortrophin ") by Organon Laboratories Ltd.

This work was carried out during the tenure by one of us, M. S., of a Fellowship of the Foundations' Fund for Research in Psychiatry.

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THE EFFECTS OF INTRACISTERNAL SARIN AND PYRIDINE-2-ALDOXIME METHYL METHANESULPHONATE IN ANAESTHETIZED DOGS

BY

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(RECEIVED OCTOBER 20, 1959)

Dogs poisoned by the anticholinesterase sarin could be saved by intravenous administration of atropine sulphate together with a suitable oxime. The central effects of intracisternal sarin were respiratory paralysis and vasomotor stimulation. The problem arose as to whether the oxime, being a quaternary nitrogen compound, could enter the brain from the blood, and could have a central action on the paralysed respiration. The methyl methanesulphonate of pyridine-2-aldoxime administered intracisternally, after sarin poisoning by the same route, was ineffective; atropine, given intravenously, was effective. The central and peripheral effects of sarin were thus reversed by the atropine-oxime therapy, the central effects by atropine, the peripheral by the oxime.

Dogs given many times the LD50 of the potent anticholinesterase sarin (isopropyl methyl-phosphonofluoridate) can be saved by the intravenous injection of atropine sulphate and the methiodide of pyridine-2-aldoxime. Atropine alone, while re-starting respiration, is incapable of maintaining it (Brown, Kunkel, Somers and Wills, 1957; Wills, Kunkel, Brown and Groblewski, 1957). The oxime alone is ineffective in re-establishing respiration. If the oxime is given 5 min. after the atropine, respiration improves within 0.5 min. which might be compatible with a central effect although quaternary nitrogen compounds penetrate the blood-brain barrier with difficulty (see Koelle and Steiner, 1956).

The experiments were undertaken to assess the central effects of sarin and of pyridine-2-aldoxime and the relative importance of central and peripheral paralysis of respiration in poisoning with sarin.

METHODS

The sarin was diluted with saline immediately before use and the volume injected was always less than 0.12 ml. Because of its greater solubility and equal efficacy (Davies and Willey, 1958), the methyl methanesulphonate of pyridine-2-aldoxime was used in the present work instead of the methiodide and was administered in aqueous solution containing 100 μg. or 1,000 μg./0.1 ml.; both freshly made and older solutions wer used, and were often tested by the copper acetate-benzidine acetate test for the presence of cyanide. The atropine sulphate solution contained 2 mg./ml., and, except as noted, was always given intravenously, 0.5 mg./kg.

The dogs were anaesthetized by pentobarbitone sodium given intravenously, enough being administered to abolish the palpebral reflex, thus giving deep surgical anaesthesia. Additional small doses were given as needed. Blood pressure was recorded on a smoked paper by a mercury manometer from a femoral or common carotid artery. The trachea was cannulated. Respirations were recorded by a Marey tambour connected with the tracheal cannula. Artificial respiration (not recorded) was given by a Starling Ideal pump. An external saphenous vein was used for intravenous injections.

The dog was placed on its belly and the neck sharply flexed. A midline incision was made with a diathermy knife from the occiput to the second spinous process. When the atlanto-occipital membrane was felt, a long needle (22 gauge) was thrust through the membrane at an acute angle. The blood pressure was observed so that any inadvertent damage to the medulla could be detected and such preparations were discarded. The penetration into the cistern was confirmed by the appearance of cerebrospinal fluid. The needle was clamped in place with towel clips and used for all intracisternal injections.

RESULTS

Effects of Pyridine-2-aldoxime Alone

Nine dogs were given pyridine-2-aldoxime, 100 or 1,000 μg. intracisternally. Only three animals

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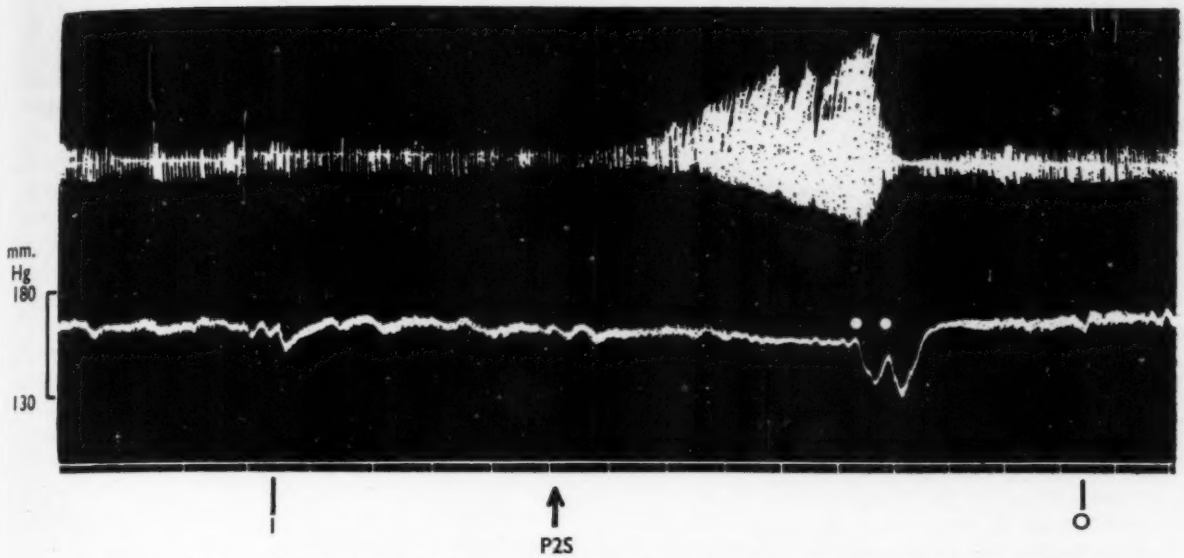

FIG. 1.—Dog, 8 kg. The effects on respiration and blood pressure of pyridine-2-aldoxime (at **P2S**) when 1 mg. was given intracisternally. The point of insertion of the needle into the cistern is marked **I**; the syringe was removed from the indwelling needle at **O**, to test fluid pressure. Upper tracing, respiration; middle tracing, blood pressure; bottom tracing, time in min. The fluctuations in blood pressure after each white dot followed injections of pentobarbitone given intravenously.

received the smaller dose, but it was repeated several times in each. At varying intervals after the first injection, slight changes of doubtful significance in respiratory rate or amplitude occurred. Subsequent doses had no visible effects, and there were no obvious changes in blood pressure. Of the six dogs which were given 1,000 μg. of pyridine-2-aldoxime, five showed some lightening of anaesthesia. The palpebral reflex returned within 1 to 8 min. after the injection; this was accompanied by severe hyperpnoea, and additional anaesthetic had to be given (Fig. 1). A second intracisternal injection of 1,000 μg. of pyridine-2-aldoxime was without effect. Intravenous or intracisternal injection of atropine after the oxime also had little effect as compared with the change observed in animals which had respiratory failure after sarin. This was to be expected, since atropine is known to have little effect on normal breathing.

Since Askew, Davies, Green and Holmes (1956) have demonstrated that many of the oximes liberate cyanide in the body and also on ageing outside the body, all the pyridine-2-aldoxime solutions used in these experiments were tested for free cyanide. None was found, nor was any detected in the cerebrospinal fluid.

After the main experiment was over, 100 to 500 μg. of sodium cyanide was injected into the cistern of several dogs. Cyanide was detected in the cerebrospinal fluid for 4 to 6 min. after this injection, and the respiratory responses were entirely different from those following pyridine-2-aldoxime injected intracisternally.

Effects of Sarin Treated with Pyridine-2-aldoxime and Atropine

The intracisternal injection of 100 μg. (average 8.62 μg./kg.) of sarin into six dogs caused a reduction in the amplitude of respiration, sometimes preceded by an increase in amplitude for 2 to 3 min.; the rate usually increased. Ultimately respiration failed. Artificial respiration was required in 10.5 ± 5.8 min. and was stopped 18 min. after the injection, but still none of the dogs breathed spontaneously. At 20 min., injection of atropine promptly restored adequate respiration. The injection of 100 μg. of pyridine-2-aldoxime intracisternally 10 min. later produced no clear respiratory response. After these two injections, respiration remained satisfactory for the remaining 3 hr. of the experiment.

The administration of sarin was also followed by a progressively rising blood pressure, which reached its peak about the time of respiratory failure (Fig. 2). Cardiovascular collapse then set in, but almost as soon as artificial respiration was begun the blood pressure returned to the previous

high level, or an even higher one. Thereafter there was a slow progressive decline in blood pressure. At 20 min., the pressure was still above the initial control level, but injection of atropine brought it at first below this level, and then quite rapidly back to about that seen initially. Subsequent injection of pyridine-2-aldoxime had little effect on the blood pressure. During the remaining 3 hr., the pressure remained at about the control level, although slow changes in pressure were present in most dogs from time to time.

There were no noteworthy differences between the responses of six dogs which received 100 μg. of sarin intracisternally, followed by artificial respiration and atropine only, and those of the dogs already considered which were given pyridine-2-aldoxime as well. The dogs in the former group were smaller and hence had a higher dose of sarin/kg. This was reflected in the shorter time to respiratory failure, 6.2 ± 4.0 min. In both groups, salivation commonly followed the intracisternal injection of sarin within 1 to 5 min. and persisted until injection of atropine.

Effects of Large Doses of Sarin and of Subsequent Treatment

Six dogs were given 25 μg./kg. of sarin intracisternally. The change in breathing was like that in the dogs which received 8.6 μg./kg., but respiratory failure occurred more rapidly in 6.0 ± 2.12 min.

The blood pressure changes were a little different. There was a rise in pressure and then some extrasystoles began to appear before the peak blood pressure was reached. Shortly thereafter severe bradycardia developed in five dogs (Fig. 3). The maximum pressure recorded by the mercury manometer was 217 mm. Hg before artificial respiration was started and 236 mm. Hg thereafter. The bradycardia continued through periods when artificial respiration was not given, with the mean blood pressure showing a rise and then a decline. The intracisternal injection of 100 μg. of pyridine-2-aldoxime 20 min. after sarin in dogs with bradycardia abolished the bradycardia completely in one dog, slightly increased the heart rate in one dog, and had no effect in three dogs. Bilateral vagotomy (2 dogs) did not relieve the bradycardia.

Eighteen min. after the sarin injection (usually 12 min. after artificial respiration began) the pump was stopped to test for the appearance of spontaneous respiration. Two min. later the pump was started and pyridine-2-aldoxime was injected intracisternally. Only two dogs showed any return of spontaneous breathing; in one of these breathing was adequate but poor, in the other failure occurred very quickly. In five dogs in this group, the intravenous injection of atropine

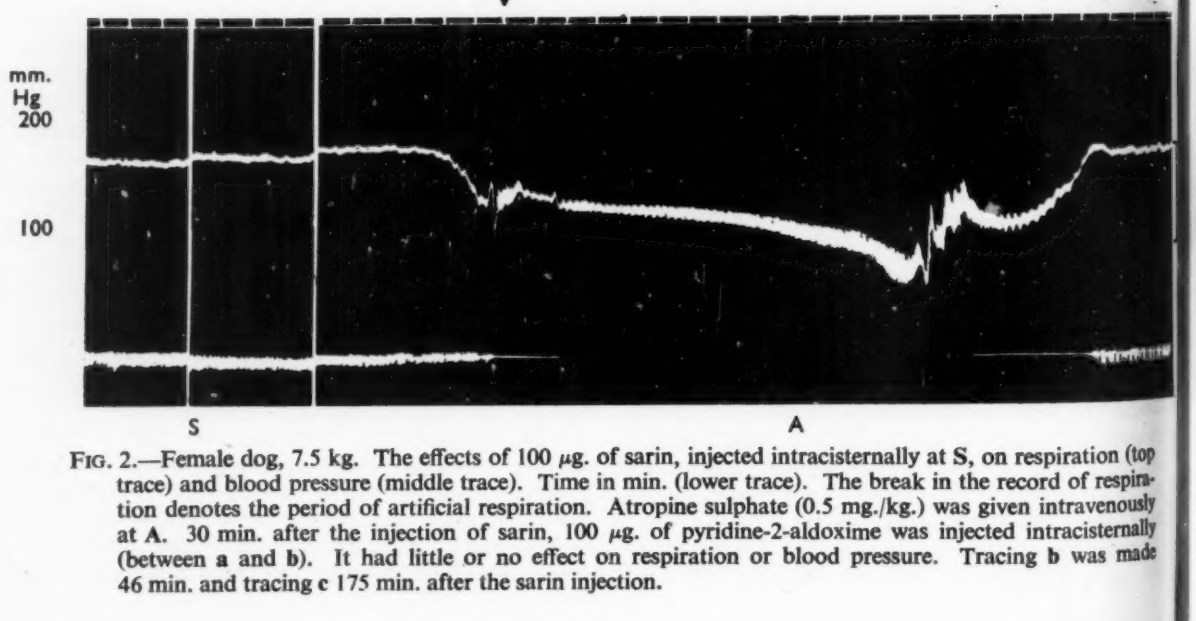

FIG. 2.—Female dog, 7.5 kg. The effects of 100 μg. of sarin, injected intracisternally at S, on respiration (top trace) and blood pressure (middle trace). Time in min. (lower trace). The break in the record of respiration denotes the period of artificial respiration. Atropine sulphate (0.5 mg./kg.) was given intravenously at A. 30 min. after the injection of sarin, 100 μg. of pyridine-2-aldoxime was injected intracisternally (between a and b). It had little or no effect on respiration or blood pressure. Tracing b was made 46 min. and tracing c 175 min. after the sarin injection.

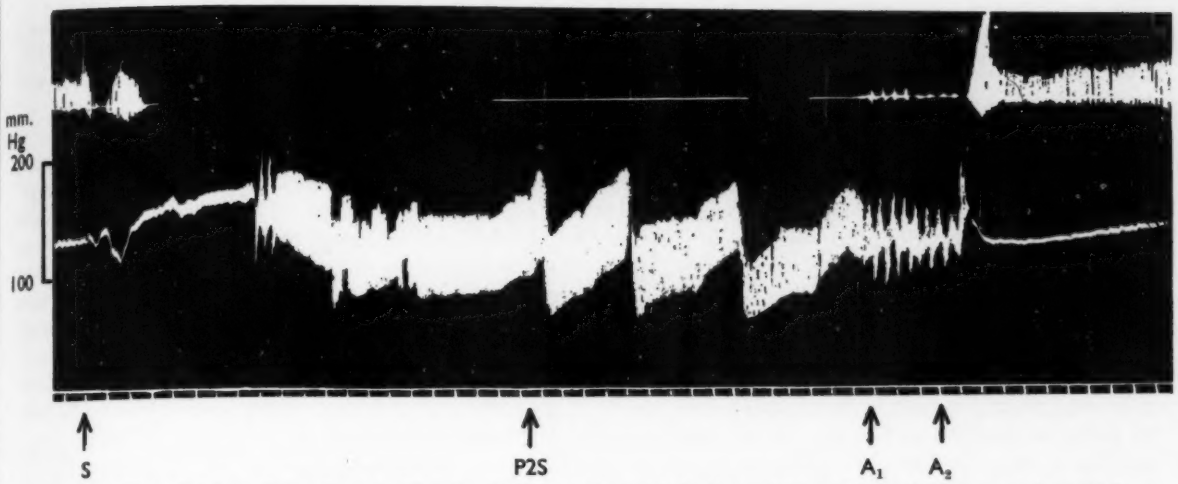

FIG. 3.—Dog, 8.5 kg. Records as in Fig. 2. An intracisternal injection of 25 μg./kg. of sarin was given at S. Note onset of extreme bradycardia about 8 min. later when salivation began. 100 μg./kg. of pyridine-2-aldoxime (at **P2S**) was ineffective. Atropine sulphate (20 μg./kg.) given intracisternally (at A_1) was partially effective. When 0.5 mg./kg. of atropine was given intravenously (at A_2) it was completely effective. The breaks in the records of respiration denote artificial respiration.

started adequate spontaneous respiration; the sixth, which was already breathing spontaneously but feebly after pyridine-2-aldoxime and artificial respiration, showed great respiratory improvement after the atropine injection. After some 30 to 60 min. when the experiments were over, no cyanide was found either in the pyridine-2-aldoxime solutions or in the cerebrospinal fluid of any of these dogs.

DISCUSSION

The doses of sarin given were all certain fatal doses, and, pending therapy, artificial respiration was essential in all dogs. Heymans, Pochet, and Van Houtte (1956) have shown that sarin does not influence the chemoreceptors. Somers and Brown (unpublished observation) have shown that doses of 10 μg./kg. of sarin intravenously had little deleterious effect on neuromuscular transmission. Hence the respiratory failure encountered after 100 μg. of sarin intracisternally must have been central in origin, as the mean dose for all dogs receiving that dose was 9.37 ± 1.97 μg./kg.

Even the larger dose of sarin, 25 μg./kg. intracisternally, produced only two signs which might have been due to peripheral action, salivation and bradycardia. The bradycardia was presumably due to some sarin being carried back with the venous blood to the heart, where it acted on the pacemaker. This effect was unaffected by bilateral vagotomy but was abolished by atropine. The salivation was probably central in origin, as it occurred in almost all the dogs which received sarin and was the only "muscarinic" effect seen in most. No other satisfactory explanation of so limited an effect is forthcoming.

Polet and de Schaepdryver (1959) found that the intracarotid injection of small doses of sarin (2 μg./kg.) stimulated, and large doses (>10 μg./kg.) depressed, respiration in dogs connected to donor-perfused heads by nervous tissue only. In the present experiments in which sarin was given intracisternally, there was always total respiratory paralysis of central origin. The conclusion of Polet and de Schaepdryver (1959) that the lethal action of sarin is not due to a primary and direct paralysis of the respiratory centre is not, therefore, tenable.

Whether given to the isolated head by intracarotid injection (Polet and de Schaepdryver, 1959) or by intracisternal injection, sarin causes a large rise of blood pressure, as reported by Dirnhuber and Cullumbine (1955). Atropine promptly abolishes this stimulation, so that the blood pressure returns to its initial level. The cardio-inhibitory centre in the medulla was not stimulated by sarin intracisternally, which confirms the results of Polet and de Schaepdryver (1959).

Pyridine-2-aldoxime, administered intracisternally, did not alter the central effects produced by sarin, but atropine appears to be an entirely adequate antidote to the central effects of sarin and its action is not altered by administration of the oxime. The failure of the oxime, injected

intracisternally, to improve respiration after sarin is difficult to explain. It is possible that pyridine-2-aldoxime does not penetrate the brain substance or the ependymal lining of the cisterna, but the lightening of anaesthesia from the larger dose raises some doubt on this point.

There are many observations which indicate that intrathecal anticholinesterases increase the quantity of acetylcholine in the brain (eserine: Bhattacharya and Feldberg, 1958; Bhawe, 1958; ethyl pyrophosphate: Metz, 1958; dyflos (diisopropylfluorophosphate): Michaelis, Finesinger, Verster, and Erickson, 1954). The role of normally-released acetylcholine in the central nervous system remains to be clarified. Whatever its role, its effects on the respiratory centre are abolished by atropine. Michaelis *et al.* (1954) concluded that atropine did not prevent the accumulation of acetylcholine, it only abolished the effects of that which was present. On other cholinergic endings, apart from those in skeletal muscle, atropine is equally effective in blocking the action of acetylcholine (see Brown *et al.*, 1957).

As de Candole, Douglas, Lovatt Evans, Holmes, Spencer, Torrance and Wilson (1953) have pointed out, respiratory failure after systemic sarin poisoning is due partly to central paralysis and partly to paralysis of neuromuscular transmission. It appears from the present results that atropine was fully effective against the central component, but it is well known that atropine in therapeutic doses has no effect on neuromuscular transmission (see tum Suden, 1958).

It is well established that pyridine-2-aldoxime leads to regeneration of cholinesterase and rapidly restores neuromuscular transmission (Brown *et al.*, 1957; Wills *et al.*, 1957; Hobbiger, 1957; Askew, 1957; Kewitz and Nachmansohn, 1957; Koelle, 1957). In the intact animal atropine can be expected to fail therapeutically against any dose of sarin sufficient to produce practically complete neuromuscular block, and any oxime known at present will fail therapeutically against any dose of sarin sufficient to produce central respiratory paralysis. Together, they are therapeutically successful against many times the LD50 of sarin (Brown *et al.*, 1957; Wills *et al.*, 1957).

Hobbiger (1957), Kewitz and Nachmansohn (1957), and Rutland (1958) have reported that therapeutically effective oximes restore the acetylcholinesterase content of the blood towards the normal level after its depression by sarin; they were unable to demonstrate a similar restoration of the brain cholinesterase. The usual explanation has been that there was an effective barrier between blood and brain. In the above experiments, it has been shown that pyridine-2-aldoxime was not effective even when an attempt was made to by-pass this barrier.

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EFFECTS OF RESERPINE, CHLORPROMAZINE AND SODIUM SALICYLATE ON THE ENZYMIC ACTIVITY OF RAT LIVER

BY

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The effects of reserpine, chlorpromazine and sodium salicylate on oxidative phosphorylation, stimulation of glutamic acid oxidation and adenosinetriphosphatase activity have been compared *in vitro* with those of 2,4-dinitrophenol using rat liver preparations. All three drugs inhibit oxidative phosphorylation, slightly stimulate glutamic acid oxidation and stimulate adenosinetriphosphatase activity at high concentrations. Reserpine has no effect on adenosinetriphosphatase activity. It is suggested that the characteristic depressant effects of reserpine on isolated tissues *in vitro* may be due to interference with production of energy by oxidative phosphorylation.

Substances which depress the formation of high energy phosphate bonds without markedly affecting oxygen consumption are known as uncoupling agents. 2,4-Dinitrophenol is a typical uncoupling agent and has been extensively used in studies on the mechanism of oxidative phosphorylation. A number of compounds chemically unrelated to one another and to dinitrophenol dissociate oxidation from phosphorylation and have been claimed to exert their biological actions through this property (Brody, 1955). Thus the antipsychotic agents, chlorpromazine and reserpine, inhibit oxidative phosphorylation in rat brain mitochondria (Abood and Romanchek, 1957) and chlorpromazine exerts a similar action in rat liver mitochondria (Berger, Strecker, and Waelsch, 1957). It has also been shown (Brody, 1956) that salicylates act as uncoupling agents, and reserpine, after *in vivo* administration, causes a significant fall in the concentrations of high energy phosphate in rat liver (Kirpekar and Lewis, 1959a).

Dinitrophenol inhibits oxidative phosphorylation (Loomis and Lipmann, 1948), stimulates the oxidation of various substrates such as glutamate, α-ketoglutarate, succinate and pyruvate + malate (Potter and Recknagel, 1951), and stimulates adenosinetriphosphatase activity (Lardy and Wellman, 1953; Myers and Slater, 1957). In the present experiments a comparison has been made between the effects of reserpine, chlorpromazine, sodium salicylate and the effects of dinitrophenol on these three systems in rat liver preparations *in vitro*.

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METHODS

A solution of reserpine (2.5 mg./ml.) and the corresponding control solvent solution (Ciba) were used and diluted as required. Aqueous solutions of chlorpromazine hydrochloride (Smith, Kline and French), sodium salicylate and 2,4-dinitrophenol were used, and diluted to give the required concentrations.

Preparation of Mitochondria

All operations were carried out at 0°. The method used for the isolation of mitochondria was essentially that described by Schneider (1948) and Schneider and Hogeboom (1950). The animals were fasted overnight to reduce the glycogen content of the liver. The liver from a freshly killed albino rat was quickly excised, washed in ice-cold 0.25 M-sucrose, blotted gently with filter paper and weighed on a torsion balance. The liver was disintegrated for 2 min. at 1,500 rev./min. in 9 volumes of cold 0.25 M-sucrose in a Potter-Elvehjem homogenizer using a plastic pestle. The mitochondrial pellet was finally suspended in 0.25 M-sucrose, so that 2.5 ml. of suspension contained the mitochondria derived from 1 g. of fresh liver. Mitochondria obtained in this manner showed a large increase in oxygen uptake if exposed to dinitrophenol or with glucose and hexokinase (Aldridge, 1957).

Measurement of Oxidative Phosphorylation

All incubations were carried out in standard Warburg flasks using the medium described by Parker

(1958) to suspend the mitochondria. Each flask contained 0.5 ml. of mitochondrial suspension, representing 200 mg. wet wt. of liver, 0.00115 mole sodium adenosine triphosphate, 0.00115 mole adenosine monophosphate, 0.125 mole potassium chloride, 0.014 mole magnesium chloride hexahydrate, 0.001 mole disodium ethylenediamine tetra-acetic acid, 0.0154 mole potassium phosphate, 0.0166 mole glycylglycine (*p*H 6.75), 0.01 mole glutamic acid as substrate, 0.06 mole glucose, 400 units of hexokinase (a preparation of type II hexokinase [Sigma Chemical Company] containing 70,000 units/g. was used) and 0.1 ml. of aqueous drug solution: for reserpine controls, 0.1 ml. of the diluted control solution was used. In the absence of drug 0.1 ml. of distilled water was used. The final flask volume was 3.0 ml., the centre well containing 0.2 ml. of 20% (w/v) KOH and a filter paper strip. The *p*H of the medium was 6.8 and the incubations were carried out at 37° in an atmosphere of air. In these experiments, glucose and hexokinase were not added to the medium in the main chamber but placed separately in the side arm. After equilibration for 10 min., the contents of the side arm were tipped and the oxygen uptake recorded for a further period of 10 min. The flasks were then immediately removed from the manometers and the reaction terminated by addition of 0.5 ml. of 60% (w/w) perchloric acid. In each experiment, the initial inorganic phosphorus content was determined in a blank flask at zero-time by deproteinizing with 0.5 ml. of 60% (w/w) perchloric acid before adding the mitochondria. The blank flask was taken through the procedure in the same manner as the others. After deproteinizing with 60% (w/w) perchloric acid the solutions were centrifuged and the inorganic phosphorus content of the supernatant fluid determined. In this way both oxygen and phosphorus uptake were measured over a period of 10 min.

Measurement of Stimulation of Oxidation

The oxidation of glutamic acid was measured using the Warburg method. The contents and the final concentration of each flask were as described in the previous section, but glucose and hexokinase were omitted. The mitochondrial suspension (0.25 ml.) added to each flask was equivalent to 100 mg. wet wt. of liver. The final volume was 3.0 ml., the *p*H 6.8 and the bath temperature 37°. The contents of the flasks were equilibrated for 10 min. and readings taken at 10 min. intervals for 1 hr.

Measurement of Adenosinetriphosphatase Activity

Adenosinetriphosphatase activity of whole liver homogenates was measured using the method of Lardy and Wellman (1953). The amount of inorganic phosphorus liberated in 10 min. from adenosine triphosphate in the presence of a liver suspension was taken as a measure of activity. Experiments were carried out in Warburg flasks at 25° in an atmosphere of air. Each flask contained 0.06 mole sodium adenosine triphosphate, 0.15 mole potassium chloride, 0.1 ml. water, reserpine control solution or the drug solution, and 0.3 ml. of liver homogenate in 0.25 M sucrose, equivalent to 100 mg. wet wt. of liver in the side arm. The total volume after tipping was 1.0 ml., the *p*H of the solution 7.2. The flasks were placed in the Warburg bath and shaken for 5 min. for temperature equilibration and the reaction started by tipping in the enzyme source. After shaking for 10 min. the flasks were immediately taken out and the reaction stopped by adding 1.0 ml. of 10% (w/w) perchloric acid. In each experiment the inorganic phosphate formed in a blank flask was estimated by deproteinizing with 1.0 ml. of 10% (w/w) perchloric acid and this value subtracted from all readings. The solutions were centrifuged and the inorganic phosphorus content of the supernatant fluid determined. Phosphate determinations were made by the method of Allen (1940).

Results

Table I shows the effects of reserpine, chlorpromazine and sodium salicylate on oxidative phosphorylation in rat liver mitochondria. In the presence of glucose and hexokinase the rate of oxygen uptake was about 2.5 times that without the acceptors. All three drugs inhibited phosphorylation without simultaneously affecting oxygen uptake. At 10^{-6} M dinitrophenol had very little effect on oxidative phosphorylation: it caused almost 50% inhibition at 10^{-5} M and 100% at 10^{-4} M. Chlorpromazine at 10^{-5} M had practically no effect on the P/O ratio (uncoupling only 5%) but began to exert its effect at 10^{-4} M, when uncoupling was about 30%, while at 5×10^{-4} M the phosphorus uptake was completely inhibited. At this same concentration chlorpromazine depressed oxygen uptake. Sodium salicylate had no uncoupling effect at 10^{-5} M, but caused 35% depression at 10^{-3} M and there was complete inhibition at 10^{-2} M. At 0.8×10^{-4} M reserpine caused 36% inhibition of phosphorylation while at 1.5 times this concentration the depression was 61%. A volume of the reserpine control solution equivalent to higher concentrations of reserpine slightly depressed the P/O ratio. Reserpine had a smaller effect on oxygen uptake, but a greater effect on phosphorus uptake than chlorpromazine. When compared with dinitrophenol, neither reserpine nor chlorpromazine nor sodium salicylate was a strong uncoupling agent. Chlorpromazine and reserpine appeared to be equipotent, but salicylate was much less potent than either.

Table II shows stimulation of the oxidation of glutamic acid in an acceptor-deficient medium. Dinitrophenol slightly stimulated it at 10^{-6} M: the effect was doubled at 10^{-5} M and increased more

TABLE I

THE EFFECT OF DINITROPHENOL, CHLORPROMAZINE, SODIUM SALICYLATE, AND RESERPINE ON OXIDATIVE PHOSPHORYLATION IN RAT LIVER MITOCHONDRIA

All concentrations are molar.

Drug	Conc.	$\Delta P\mu$ Mole (±S.E.)	$\Delta O\mu$ Atoms (±S.E.)	P/O (±S.E.)	% Uncoupling
Control	—	25·82 (±0·52)	9·5 (±0·28)	2·76 (±0·05)	—
Dinitrophenol ..	1×10^{-6}	19·39 (±0·16)	7·94 (±0·10)	2·43 (±0·05)	12
	1×10^{-5}	12·28 (±0·46)	8·80 (±0·47)	1·40 (±0·11)	49
	1×10^{-4}	0·00	6·90 (±0·12)	0·00	100
Chlorpromazine ..	1×10^{-5}	20·25 (±0·27)	7·74 (±0·11)	2·61 (±0·00)	5
	1×10^{-4}	17·22 (±1·60)	8·67 (±0·60)	1·96 (±0·06)	29
	5×10^{-4}	0·00	2·14 (±0·05)	0·00	100
Sodium salicylate ..	1×10^{-5}	27·52 (±1·10)	10·05 (±0·02)	2·71 (±0·10)	2
	1×10^{-4}	16·44 (±0·86)	7·63 (±0·20)	2·16 (±0·17)	22
	1×10^{-3}	12·46 (±1·40)	6·87 (±0·14)	1·79 (±0·17)	35
	1×10^{-2}	0·00	5·96 (±0·07)	0·00	100
Reserpine control ..		17·91 (±1·6)	7·94 (±0·58)	2·25 (±0·10)	—
Reserpine	$1·6\times10^{-5}$	22·51 (±0·16)	11·25 (±0·18)	2·00 (±0·04)	11
	$0·8\times10^{-4}$	12·44 (±0·29)	8·68 (±0·58)	1·44 (±0·09)	36
	$1·2\times10^{-4}$	4·51 (±1·27)	4·88 (±0·31)	0·87 (±0·23)	61

TABLE II

EFFECTS OF DINITROPHENOL, RESERPINE, CHLORPROMAZINE, AND SODIUM SALICYLATE ON OXYGEN UPTAKE IN AN ACCEPTOR-DEFICIENT MEDIUM

Control values (1 hr.) are taken as 100%. All concentrations are molar.

Drug	Conc.	% Oxygen Uptake
Dinitrophenol ..	1×10^{-6}	115
,, ..	1×10^{-5}	200
,, ..	1×10^{-4}	265
Reserpine ..	$1·6\times10^{-5}$	115
,, ..	$0·8\times10^{-4}$	117
,, ..	$1·2\times10^{-4}$	83
Chlorpromazine	1×10^{-5}	107
,,	5×10^{-5}	113
,,	1×10^{-4}	124
,,	5×10^{-4}	6
Sodium salicylate	1×10^{-5}	104
,, ,,	1×10^{-4}	119
,, ,,	1×10^{-3}	108
,, ,,	5×10^{-3}	100
,, ,,	1×10^{-2}	100

than 2.5 times at 10^{-4} M. Reserpine at 1.6×10^{-5} M and 0.8×10^{-4} M stimulated oxygen uptake 15%. The reserpine control solution had virtually no effect on glutamic acid oxidation. When used with liver slices, the control solution significantly stimulated oxygen uptake (Kirpekar and Lewis, 1959a). Chlorpromazine had no effect at 10^{-5} M, caused approximately 25% stimulation at 10^{-4} M and almost complete inhibition at 5×10^{-4} M. Sodium salicylate caused slight stimulation at 10^{-4} M, but even at very high concentrations (10^{-3} to 10^{-2} M) it had very little effect. None of the drugs tested had so marked an effect on glutamic acid oxidation as dinitrophenol. Dinitrophenol apparently compensates for the deficiency of glucose and hexokinase, since in its presence oxygen uptake was even greater than in the presence of such an acceptor.

Table III shows the effects of chlorpromazine, reserpine, sodium salicylate and dinitrophenol on liver adenosinetriphosphatase activity. Dinitrophenol had a very high stimulant activity at 3×10^{-4} M, but at lower concentrations it was less effective and at 10^{-6} M inactive. Chlorpromazine had no effect at 10^{-5} M and 10^{-4} M, but at 10^{-3} M had about half the effect of dinitrophenol (3×10^{-4} M). Reserpine at the concentrations tested had no stimulant effect upon adenosine-

TABLE III

EFFECTS OF DINITROPHENOL CHLORPROMAZINE, RESERPINE, AND SODIUM SALICYLATE ON LIVER ADENOSINETRIPHOSPHATASE ACTIVITY

All concentrations are molar.

Drug	Conc.	μmole Inorganic Phosphate Liberated ±(S.E.)
Control ..	—	0·87 (±0·05)
Dinitrophenol ..	3×10^{-6}	1·10 (±0·07)
,, ..	3×10^{-5}	2·59 (±0·12)
,, ..	3×10^{-4}	8·35 (±0·10)
Chlorpromazine	1×10^{-5}	1·04 (±0·16)
,, ..	1×10^{-4}	1·04 (±0·08)
,, ..	1×10^{-3}	3·67 (±0·02)
Reserpine ..	$1{\cdot}6\times10^{-5}$	1·04 (±0·14)
,, ..	$0{\cdot}8\times10^{-4}$	0·37 (±0·13)
,, ..	$1{\cdot}2\times10^{-4}$	0·22 (±0·09)
Sodium salicylate	1×10^{-4}	1·32 (±0·00)
,, ,,	1×10^{-3}	2·15 (±0·21)
,, ,,	1×10^{-2}	5·97 (±0·19)

triphosphatase activity, but caused slight inhibition at higher concentrations. Sodium salicylate enhanced adenosinetriphosphatase activity (6.00 μ mole of phosphate at 10^{-2} M); had little stimulant effect at 10^{-3} M and none at 10^{-4} M. It was interesting to note that the concentrations of dinitrophenol which produced maximum stimulation of oxygen uptake and of adenosinetriphosphatase action were similar. This does not, however, appear to be true of the other compounds investigated.

Table IV summarizes the results and gives comparative data on the four compounds studied.

DISCUSSION

Dinitrophenol, which is a typical uncoupling agent, powerfully stimulates adenosinetriphosphatase activity and glutamic acid oxidation, and causes marked inhibition of phosphorylation without affecting oxygen uptake. Reserpine, chlorpromazine and sodium salicylate all inhibit oxidative phosphorylation, and with higher concentrations both oxygen and phosphorus uptake are depressed. There is some difference of opinion regarding the action of chlorpromazine on rat brain mitochondria. Abood and Romanchek (1957) have shown that chlorpromazine depresses oxidative phosphorylation, while Berger, Strecker and Waelsch (1957) did not confirm this but reported depression of P/O ratios in rat liver mitochondria and thereby stressed the difference in the metabolic behaviour of mitochondria from the brain and liver. Our observations support their finding that chlorpromazine inhibits phosphorylation in rat liver mitochondria. Reserpine has been reported by Abood and Romanchek (1957) to be an effective uncoupling agent in rat brain mitochrondria, and our results show that it is an uncoupling reagent in rat liver mitochondria. It appears therefore that, as far as reserpine is concerned, brain and liver mitochondria behave similarly. It is also possible that reserpine, which has marked effects on the central nervous system, may possess a preferentially higher affinity for brain than for liver cells. Reserpine has also been reported by the same authors in *in vivo* studies to have an uncoupling effect in preparations of mitochondria

TABLE IV

COMPARATIVE CONCENTRATIONS OF DINITROPHENOL, RESERPINE, CHLORPROMAZINE, AND SODIUM SALICYLATE AFFECTING THE P/O RATIO, OXIDATION AND ADENOSINETRIPHOSPHATASE ACTIVITY IN RAT LIVER

All concentrations are molar.

Experiment	Dinitrophenol	Reserpine	Chlorpromazine	Sodium Salicylate
50% inhibition of P/O ratio	1×10^{-5}	1×10^{-4}	2×10^{-4}	$2{\cdot}5\times10^{-3}$
Maximum stimulation of oxidation ..	1×10^{-4}	$0{\cdot}8\times10^{-4}$	1×10^{-4}	1×10^{-4}
Maximum stimulation of adenosinetriphosphatase activity	3×10^{-4}	None	1×10^{-3}	1×10^{-2}

from the region of the basal ganglia. It has been shown recently by Desci and Mehes (1959) that the tranquillizing agents benactyzine and meprobamate also have an uncoupling effect which is exerted more effectively on preparations from the same regions.

At a concentration of 10^{-4} M dinitrophenol markedly stimulates the oxidation of glutamic acid. The other drugs are inactive, and although they inhibit oxidative phosphorylation in certain concentrations they do not possess the property of stimulating oxidation. Dinitrophenol stimulates oxidation markedly and at the same time inhibits phosphorylation. Chlorpromazine (10^{-3} M) and sodium salicylate (10^{-2} M) stimulate adenosinetriphosphatase activity considerably, but there appears to be no relation between the dose causing maximum stimulation of adenosinetriphosphatase activity and the effect on oxygen uptake. Reserpine, on the other hand, has little stimulant effect on oxidation (10^{-4} M) but at the same concentration has a slight inhibitory effect on adenosinetriphosphatase activity.

The effects of chlorpromazine and reserpine, like those of thyroxine, may be due to intracellular physical processes such as the swelling of the mitochondria. Thyroxine causes the mitochondria to swell (Dickens and Salmony, 1956; Tapley, Cooper, and Lehninger, 1955) and the uncoupling effect that it produces is attributed to this property rather than to its interaction with the enzymes concerned with oxidative phosphorylation. It would be interesting to see whether chlorpromazine or reserpine cause swelling of mitochondria although the general pharmacological properties of reserpine and chlorpromazine are quite different from those of thyroxine.

Chlorpromazine and salicylates react with ferric salts. Chlorpromazine when mixed with ferric chloride solution is oxidized and produces a brilliant red colour. Our recent observations (Kirpekar and Lewis, 1959b) of the apparent formation of a hydrallazine-iron complex and the observation of Fels and Kaufmann (1959) that chlorpromazine possesses a specific affinity for iron make it possible that chlorpromazine influences phosphorylation at the cytochrome-cytochrome oxidase level. But a few experiments carried out with crystalline catalase (an iron containing enzyme) have not shown chlorpromazine to have an inhibiting effect even when used at very high concentrations (1.8×10^{-2} M). Salicylates are chelating agents, but reserpine has not so far been reported to form chelation complexes.

In intact animals reserpine is known to exert its characteristic effects rather slowly. Gillis and Lewis (1956) and Kirpekar and Lewis (1958) have shown that reserpine depresses skeletal muscle, the smooth muscles of intestine, uterus and arteries, and that it also depresses heart muscle. This effect may be due to its uncoupling action (Kirpekar and Lewis, 1959a). Born and Bülbring (1955) have shown that dinitrophenol in high concentrations decreases the tension and spontaneous movements of the taenia coli muscle. Moreover, when the dinitrophenol-treated muscle is stimulated with histamine, the tension increases but is not maintained although histamine is still present. Anoxia also depresses tissue activity. There is thus some evidence that, under the conditions of our experiments, reserpine and chlorpromazine act like dinitrophenol, but the absence of a reserpine effect on adenosinetriphosphatase activity shows that chlorpromazine and reserpine have different modes of action from one another and from dinitrophenol, a view supported by pharmacological observations. Sodium salicylate has no antipsychotic action of the type exerted by reserpine and chlorpromazine, but it has some marked effects on the central nervous system: it can, for example, cause nausea and vomiting, analgesia, antipyresis and respiratory stimulation. It has also been shown to inhibit catalase. The effects of sodium salicylate on the metabolic rate (Cochran, 1952) and in causing depletion of liver glycogen (Sproull, 1954) and hyperglycaemia (Bornstein, Meade, and Smith, 1952) may have some bearing on its effect on oxidative phosphorylation, but in man even large doses of salicylate do not produce the effects typical of an uncoupling agent.

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A COMPARISON OF THE KALLIKREIN–KININ SYSTEM IN SHEEP AND DOGS

BY

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Three preparations of kallikrein, made from the urine of man, dog and sheep, all formed active kinins when incubated with the plasma of sheep or dogs. The six kinins could not be distinguished from one another by parallel assays on guinea-pig ileum, rat uterus and rat duodenum; they are thought to be identical, or nearly so. As judged by the amount of kinin produced, a sample of dog plasma appeared to contain about 4·3 times as much substrate as a sample of sheep plasma. This ratio did not depend on the kallikrein used. Attempts to compare the three kallikreins gave inconsistent results which may be due to the presence in the preparations of kallikrein of other enzymes acting on the same substrate. Sheep plasma does not form kinins on contact with glass.

Kallikrein is a non-dialysable substance extractable from urine, pancreas, pancreatic juice, salivary glands and saliva of many animals (Frey, Kraut, and Werle, 1950). Its actions depend, partly at least, on its ability to react with plasma proteins to produce a smooth muscle stimulating substance called kallidin (Frey *et al.*, 1950; Werle, 1955) or plasma kinin (Horton, 1958; Horton, 1959a; Margolis, 1958; Lewis, 1958; Hilton and Lewis, 1958).

The reaction is complicated by the presence in plasma of substances, kallikreinase and kininase, capable of destroying kallikrein and kinin. This report is concerned primarily with a comparison of the entire system, urinary kallikrein, plasma kallikrein substrate, kallikreinase and kininase in two mammalian species.

METHODS

Rat Uterus.—The uteri from virgin rats (150 to 250 g.) were suspended in de Jalon solution in a 3 ml. bath at 30°.

Guinea-pig Ileum.—The terminal segments of ileum of guinea-pigs (180 to 300 g.) were suspended in Tyrode solution in a 3 ml. bath at 37°. Atropine sulphate (1 mg./l.) was used when the spontaneous movement was not otherwise controllable.

Rat Duodenum.—The proximal segments of duodenum of rats (150 to 250 g.) were suspended in de Jalon solution in a 3 ml. bath at 30 to 37°. The tissue was stored at 4° for 2 to 3 hr. before use.

The dose interval was usually 3 min. The bath was washed out by continuous upward displacement and overflow. The preparations seemed most responsive 30 sec. after this slow flow was stopped, and the substances were injected at this time. The inflow was started 15 sec. after injection in the experiments with the duodenum, and 1 min. with the ileum and uterus preparations.

Preparations of Human, Sheep and Dog Urinary Kallikreins.—Urine from male humans, a Clun Forest wether sheep and mongrel dogs of both sexes was collected under toluene and frozen until use. The procedure for preparing crude kallikrein was that described by Gaddum and Horton (1959) with the exception that the urine was concentrated by placing it in dialysis bags and hanging them before a fan and heat source, rather than by the use of a climbing film evaporator. The concentrated urine was dialysed and the kallikrein precipitated with acetone.

Preparation of Kinins.—The procedure for making the six kinins used in this study was that described by Horton (1958) and Gaddum and Horton (1959). Urinary kallikrein from three sources (human, sheep and dog) (1 mg. kallikrein per 10 ml. plasma) was incubated with acid-treated plasma from two sources (sheep and dog) for 2 hr. at 37°. This acid-treatment consisted in incubation at *p*H 2 and 37° for 10 min.; this was found by Horton (1958, 1959b) to inhibit kallikreinase and kininase without affecting kinin formation. After incubation and neutralization the mixture was poured into boiling ethanol, and the kinin prepared by drying the alcoholic extract.

The first letter of the kinin notation system used in this paper refers to the source of kallikrein, while the second letter refers to the source of the substrate. Thus HD is the kinin produced when human urinary

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kallikrein reacts with dog plasma substrate; this was the preparation used as a standard.

Kallikrein Substrate Assay.—The procedure outlined for the preparation of the kinins was designed for maximum kinin formation. Excess kallikrein and an incubation period about twice as long as necessary for the reaction to go to completion were used. The limiting factor in the reaction was the substrate concentration of the plasma. By using the same kallikrein with different substrates, preparing the kinins thus formed and assaying them against a standard kinin (HD), a measure of the relative substrate concentration was obtained.

Results

The experiments illustrated in Fig. 1 showed, by a new method, that sheep resemble other animals in that their urine contains kallikrein and their plasma contains both kallikrein and a kallikreinase which is destroyed by incubation at *p*H 2 and 37° for 10 min. as recommended by Horton (1958, 1959b). Three samples of a preparation of sheep urinary kallikrein (2 mg./10 ml. of the mixture) were incubated at 40° for 24 hr. in dialysis bags with (*A*) de Jalon solution, (*B*) normal sheep plasma and (*C*) acid-treated sheep plasma; and allowed to dialyse for 24 hr. against 10 volumes of de Jalon solution, which was changed once during the dialysis. Kinin was presumably formed during this process and mostly dialysed away; the contents of each bag was then incubated at room temperature with 10 to 25 ml. of dialysed acid-treated sheep plasma, and serial samples were taken from each incubation mixture and tested every 9 min. on an isolated rat uterus preparation. The tracing shows the effects of samples at 45 and 54 min. after mixing.

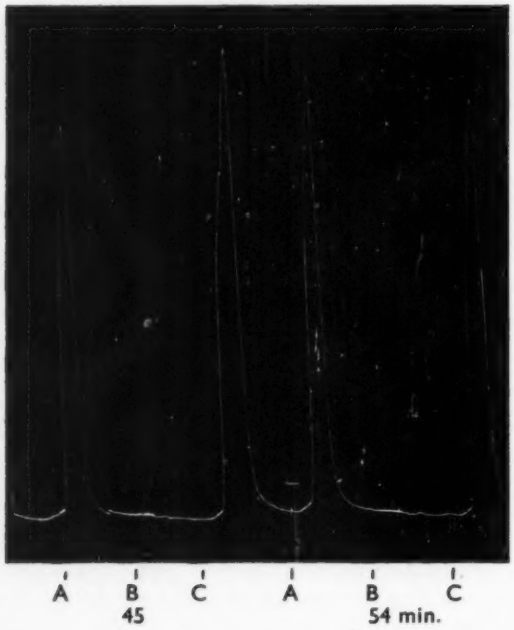

FIG. 1.—Isolated rat uterus. Presence of kallikreinase in sheep plasma and its inhibition by acid treatment. Three samples of sheep kallikrein were incubated with (A) de Jalon solution, (B) normal sheep plasma and (C) acid-treated sheep plasma, and the kinin dialysed away. They were then all incubated with acid-treated plasma and tested at intervals as indicated. Sample B now contained no kallikrein.

The kallikrein incubated with normal sheep plasma (*B*) was completely inactivated, presumably by kallikreinase. The kallikrein incubated with acid-treated sheep plasma (*C*) was spared and formed kinin when added to fresh plasma. This demonstrates that acid-treatment inhibits kallikreinase, and provides evidence that kallikrein is an enzyme. The kallikrein had presumably formed kinin during the dialysis, but had not itself been inactivated in the process; it behaved like a catalyst.

In another experiment, kallikrein, which had been incubated overnight at room temperature with acid-treated plasma in a dialysis bag, was compared with kallikrein which had been incubated with de Jalon solution. After these preliminary incubations they were both incubated with acid-treated dog plasma for 2 hr. and kinin was then extracted with boiling alcohol. The amount of kinin formed by the sample exposed to plasma was estimated to be 80% of the amount formed by the other sample. This result indicates that the acid treatment had not completely destroyed both kallikreinase and kininase.

Fig. 2 is the tracing of an experiment designed to illustrate the presence of kininase in sheep plasma and its destruction by acid treatment. Three samples of kinin made from sheep kallikrein and sheep plasma were incubated at room temperature with (*A*) de Jalon solution, (*B*) normal sheep plasma, and (*C*) acid treated sheep plasma. Serial samples were taken every 3 min. and tested on isolated rat uterus. The tracing shows the progressive destruction of kinin by normal sheep plasma, but not in the other two samples.

Table I gives the results of parallel assays against HD kinin of the kinins produced by allowing three urinary kallikreins (human, sheep and dog) to react with two substrate sources (sheep and dog). The index of discrimination (Gaddum, 1955) is a measure of the dissimilarity of two substances. It is the ratio of estimates of the activity of one substance when compared with

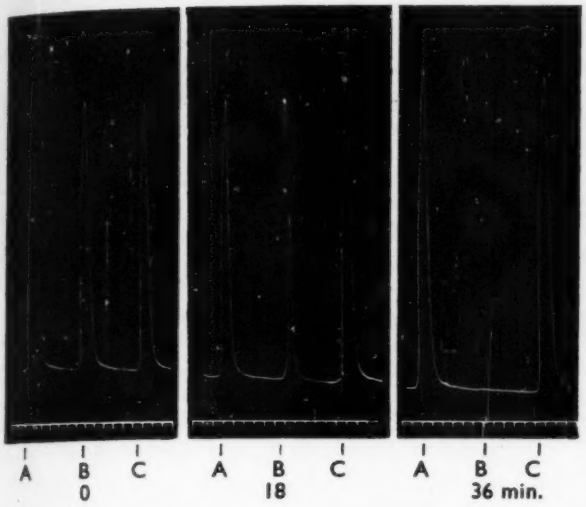

FIG. 2.—Presence of kininase in sheep plasma and its inhibition by acid treatment. Kinin incubated with normal sheep plasma is destroyed (B), while kinin incubated with de Jalon solution (A) or acid-treated plasma (C) is preserved.

TABLE I

COMPARATIVE ACTIVITY OF THE KININS

Activity of 1 mg. of various kinins in terms of mg. HD kinin. Means and ranges of 4 six-point assays. The first letter in the first column denotes the source of kallikrein and the second letter the source of substrate. S—sheep, H—human, D—dog.

Kinin	Guinea-pig Ileum	Rat Uterus	Rat Duodenum	Index of Discrimination	
				RU/GPI	RD/RU
SD	1·9 (1·3–2·8)	2·1 (1·5–3)	1·9 (1·4–2·5)	1·1	0·9
HD	1·0	1·0	1·0	1·0	1·0
DD	0·33 (0·33–0·33)	0·31 (0·29–0·35)	0·38 (0·33–0·5)	0·9	1·2
SS	0·46 (0·33–0·53)	0·66 (0·33–1·2)	0·59 (0·25–1·3)	1·4	0·9
HS	0·14 (0·083–0·25)	0·23 (0·20–0·3)	0·25 (0·22–0·28)	1·6	1·1
DS	0·095 (0·062–0·17)	0·056 (0·04–0·067)	0·095 (0·071–0·17)	0·6	1·7

another on two different smooth muscle preparations. An index near unity indicates that the preparations used are unable to discriminate between the two agents being tested. This method clearly discriminates between kinins on the one hand, and substance P or angiotensin on the other (Gomes, 1955).

In the case of the kinins, they have all been assayed against HD kinin on three smooth muscle preparations. The indices of discrimination calculated from the parallel assay figures indicated that no qualitative differences between the various kinins could be detected.

In these experiments there was excess of kallikrein, and incubation was continued until the reaction was complete. In these circumstances it would be expected that the amount of kinin produced would be directly proportional to the concentration of substrate. Table II shows 9 estimates, based on this assumption, of the ratio of the concentration of substrate in the sample of dog plasma to that in the sample of sheep plasma. These ratios were calculated from the figures given in Table I. The differences between these 9 estimates are within the error of the methods used. There is no evidence that the ratio depends either

TABLE II

SUBSTRATE IN THE PLASMA OF SHEEP, HUMAN AND DOG

Nine estimates of the ratio of the concentration of kallikrein substrate in a sample of dog plasma to that in a sample of sheep plasma calculated from the results shown in Table I. Thus 4·1 = 1·9/0·46.

Kallikrein	Test Organ		
	Guinea-pig Ileum	Rat Uterus	Rat Duodenum
Sheep ..	4·1	3·2	3·2
Human ..	7·1	4·3	4·0
Dog ..	3·5	5·5	4·0

on the kallikrein or on the method of assay. The simplest interpretation of these results is that dog plasma contains the same substrate as sheep plasma, but in 4.3 times the concentration.

Similar calculations of ratios can be made from the figures in Table I to compare the different kallikreins with one another. In this case it would not be expected that the ratio would give a direct estimate of the concentration of kallikrein, since there was always excess of kallikrein. On the simplest assumptions the amount of kinin produced would be independent of the kallikrein used. All the figures in the top half of Table I would then be equal to one another, and all the

figures in the bottom half would also be equal. This is clearly not the case; the figures show consistently that sheep kallikrein produced more kinin than human kallikrein, and that dog kallikrein produced less.

Attempts were made to compare the three kallikreins by the method of Horton (1958, 1959a), in which the kinin formed by small doses of kallikrein in a short time is measured, but the results were difficult to interpret. When acid treated dog plasma was incubated for 10 min. with 10 to 100 μg. of each of the three kallikreins, the results seemed to show that the kallikrein made from dog urine was the most active. In a similar experiment with dog plasma diluted 1:10, the human kallikrein appeared to be the most active. These results were both unexpected, since the experiments shown in Table I had suggested that sheep kallikrein was the most active of the three.

It was thought that these results might depend on the presence of kininase in the preparation of kallikrein. This would not be surprising since there is evidence that urine may contain kininase (Frey, Kraut, and Werle, 1950; Jensen, 1958; Horton, 1959a). In order to test this possibility, the standard preparation of kinin (HD) was incubated with the three kallikreins and the fluid tested on rat uterus. This experiment showed that the preparation of sheep kallikrein caused a gradual destruction of kinin, and that the other two kallikreins had a smaller effect of the same kind. This finding does not account for the observed difference in the yields of kinin, but it does draw attention to a possible source of error in experiments of this kind.

Armstrong, Jepson, Keele, and Stewart (1955) reported that dog plasma is not activated by contact with glass to produce a smooth muscle stimulating substance. In the course of these experiments we have noted that sheep plasma similarly does not appear to be activated by contact with glass.

Discussion

These results suggest that the same kinin is formed by the action of human, dog or sheep kallikrein on either dog or sheep substrate, and that there was no difference between these substrates except in concentration. There were clear differences in the amount of activity formed by the different kallikreins. This might be taken as evidence that the kallikreins are different, but there was no evidence that kallikreins have a different action on the substrate obtained from the same species from that which they have on substrates obtained from other species.

A possible explanation of the results depends on the assumption that the preparations of kallikrein contain another enzyme which competes with kallikrein for the same substrate, but does not form a peptide which stimulates plain muscle. Another explanation is suggested by the evidence of Lewis (1959) that urine may contain two different kinin-forming enzymes. There are other possible explanations. It is clear that more evidence is needed.

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Brit. J. Pharmacol. (1960), **15**, 185.

UNSPECIFIC DRUG ACTION. THE EFFECTS OF A HOMOLOGOUS SERIES OF PRIMARY ALCOHOLS

BY

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Experimental results relating to the unspecific depressant action of normal primary alcohols from methanol to octanol on four separate biological systems are presented. It was found that the log-concentration action curves of alcohols on all four systems were straight over most of their range and, for any one system, parallel throughout the series. With arithmetic increase in the alcohol chainlength the concentration required to produce a given effect diminished logarithmically. The rate of this decrease varied in different biological systems, and was always less than the rate of decrease of solubility with chainlength. In two of the systems investigated alcohols beyond octanol failed to show any activity (cut-off phenomenon). The implications of these findings are discussed, with reference to the mechanism of action of unspecific depressants. Ferguson's principle of using thermodynamic activity instead of concentration as an index of activity was applied to the present results. In an appendix, the results are compared with predictions according to Mullins' hypothesis of narcotic action, and found not to agree well.

A wide range of simple organic compounds can exert qualitatively identical depressant actions on many different organisms. The striking absence of chemical specificity in the compounds tested led to the theory that physical rather than chemical properties govern the activity of the compounds. This approach resulted in the formulation of two theories of narcotic action, namely the Overton-Meyer theory and the Traube theory.

Meyer, in 1899, proposed that narcosis results from the drug dissolving in a fatty phase within the cell. This theory predicts that the narcotic potency of a drug will depend on its fat/water partition coefficient, and also that lipoid-rich cells will be most readily affected. Overton (1901) published data showing a striking correlation between narcotic potency (in tadpoles) and olive-oil/water partition coefficient for a wide range of compounds. In 1935 K. H. Meyer and Hemmi found a correlation between narcotic potency of vapours in mice and the oleic-acid/water partition coefficient of the compound. This was held to support the Overton-Meyer theory. Traube (1904) correlated narcotic potency with the activity of lowering surface tension at an air/water interface. Meyer and Hemmi (1935) found that this correlation does not hold with vapours. On the other hand, Warburg (1921) considered that Traube's air/water interface was a rather unlikely model of any cell component, and measured the adsorption of narcotics at a charcoal/water interface, and found a good correlation with narcotic activity. He postulated inhibition of an enzyme by adsorption of narcotic at some cellular interface as a mechanism of narcosis.

Concurrently with these investigations of narcosis, investigations of the bactericidal activity of organic compounds were carried out, mainly by Tilley and Schaffer (1926, 1928), who investigated the relationship between bactericidal potency and chainlength in homologous series. They found that with an arithmetic increase in chainlength, the equitoxic concentration fell logarithmically up to a certain chainlength (the cut-off point) at which it rose rapidly and very soon reached the solubility limit. The logarithmic fall in equitoxic concentration ran approximately parallel to the fall in solubility with chainlength, but was always slightly less steep. Tilley and Schaffer did not seek to correlate potency with any particular physical property.

Also at this time, new insecticides were being sought. Holt (1916) and Moore (1917) related insecticidal potency with boiling point, and Moore put forward the generalization that potency increases with boiling point up to a certain boiling point, above which compounds cease to be toxic. Boiling point is a way of expressing the distribution of a compound between its liquid and vapour phases.

Ferguson (1939) suggested that the use of distribution coefficients be avoided by measuring potency in terms of the thermodynamic activity of the depressant agent in the solution, instead of its concentration. Since the thermodynamic activity of a substance is by definition equal in all phases of a system at equilibrium, the problem of distribution coefficients between numerous different phases within a cell, any of which might be the biophase in which the drug exerts its pharmacological effects, is by-passed. Using this new approach, Ferguson recalculated the data of earlier workers, and also measured the insecticidal potency of many different vapours. A number of generalizations emerged. For nearly all the compounds tested the thermodynamic activity required to produce a narcotic or toxic effect was 0.1 or more. In a few striking exceptions the activity required was much lower (e.g., ammonia, hydrocyanic acid), and the pharmacological action was also quite different. Hence these compounds may be excluded from the wide group of unspecific depressants. Ferguson also found that within an homologous series the thermodynamic activity required to produce a given effect increases steadily with chainlength until it reaches unity (the activity of a saturated solution). Beyond this point the effective thermodynamic activity is unattainable and the cut-off noticed by earlier workers is thereby explained without having to postulate a sudden change of properties at a particular chainlength.

On the other hand Brink and Posternak (1948), investigating depression of conduction in isolated nerve preparations by homologous series of narcotics, found that the equipotent thermodynamic activity showed no progressive rise throughout a homologous series, but rose sharply to unity at a certain chainlength.

Mullins (1954) devised a more direct approach to the problem. He started with the hypothesis that narcotics act by dissolving in some particular phase of the cell (probably the membrane), and that equal narcotic effects are produced when an equal volume fraction of this phase is occupied by narcotic molecules. By making a number of assumptions and approximations concerning the physico-chemical properties of the biophase and its interaction with the narcotic, Mullins was able to predict equipotent thermodynamic activities within a homologous series, and he presents experimental results which show reasonable agreement with these predictions.

The present work was undertaken with the following aims in view.

1. To gain comparative data on the action of a single homologous series (normal primary alcohols) on several different biological systems. This is the converse of the approach usually adopted in the past, and it was interesting to see whether the slight differences between homologous series patterns were also evident when the same series was used on different systems.

2. To throw more light on the mechanism of cut-off, as argued by Ferguson on the one hand and Brink and Posternak on the other.

3. To try to determine the level of organization at which alcohols exert their action, by investigating a sequence of phenomena ranging from mobility of whole organisms to oxygen consumption of isolated tissues.

4. To provide further material on which to test Mullins' hypothesis.

Methods

Paramecium Immobilization

The concentration of different alcohols required to produce the same degree of immobilization in a population of paramecia was measured.

0.2 ml. of a suspension of paramecia in water (containing 100 to 200 organisms) was placed in a small flat perspex cup. Six such cups fitted into the revolving stage of a low-power binocular microscope, so that each could be swung rapidly into the field of view. At a noted time a volume of alcohol solution (0.05 to 0.2 ml.) calculated to give a suitable final concentration was added from a syringe. At known intervals after addition of alcohol, the cups were examined in turn, and counts of the number of immobile organisms (which sink conveniently to the bottom and are quite easily distinguishable) were made. Counts of each cup were made at 5 min. intervals, until the count became steady. When such an equilibrium had been reached (usually after about 15 min.) a drop of strong phenol solution was added to each cup. This immobilized all the organisms and the total number in each cup was counted. The fraction of the total number of organisms immobilized at equilibrium was taken as the response to a particular alcohol concentration.

Gut Contractility

Experiments were carried out on the guinea-pig ileum, suspended in oxygenated Tyrode solution in a 1 ml. organ bath maintained at 32 to 34°.

Contractions elicited by additions of acetylcholine to the bath were recorded by means of a light frontal-writing lever exerting a tension of about 0.3 g. on the ileum. Alcohols dissolved in the Tyrode washing fluid were applied to the preparation and the assays carried out with an automatic assay apparatus (Boura, Mongar and Schild, 1954).

In one set of experiments the ileum was stimulated electrically. The stimulus was supplied from a square-

pulse stimulator, and applied to the preparation between its attachments, which were made with fine wire in place of thread.

Tissue Oxygen Consumption

Experiments were carried out on chopped guinea-pig lung. The animal was killed by a blow on the back of the neck, and the lungs removed and washed in Tyrode solution. The lungs were then chopped into particles 1 mm. square in cross section, using a MacIlwain tissue chopper. 0.2 ml. samples were prepared, using the sampler described by Mongar and Schild (1957).

A few samples were dried and weighed. Their dry weight was about 3 mg. and varied very little between samples from the same animal.

Oxygen consumption was measured by Warburg's direct method. The main compartment of each flask contained 3.0 ml. of Tyrode solution (containing phosphate buffer in place of bicarbonate) and the lung particles, and, in most of the experiments, the alcohol. The central well contained 0.4 ml. of 10% sodium hydroxide solution absorbed in a filter paper roll. In some experiments with water-miscible alcohols, the alcohol was placed in the side-arm, and added only after the control rate of oxygen consumption had been established. In most cases, however, it was necessary to add the alcohol to the main compartment right from the start, and to compare the oxygen uptake with that of separate control flasks.

Anaphylactic Histamine Release

The method described by Mongar and Schild (1957) was used.

Guinea-pigs were sensitized by injecting a solution of 2% egg albumen and 0.5% phenol in normal saline, 1 ml. being given subcutaneously and 1 ml. intraperitoneally. The animals were used 3 to 10 weeks later.

The lungs were chopped as before, and washed for at least 20 min. in 500 ml. of Tyrode solution, in order to remove as much as possible of the histamine released from damaged cells. 0.2 ml. samples were prepared as before, and each sample was placed in a 5 ml. beaker containing 1 ml. of Tyrode solution, or Tyrode plus inhibitor, and a round glass bead was added to help stirring. The beakers were fitted into a shaking rack in a water-bath at 37°, and left for 15 min. 0.1 ml. of 1% egg albumen solution was then added to each (with the exception of the blanks, to which Tyrode was added) and after a further 15 min. shaking the supernatant was sucked off with a filter-tipped pipette. The supernatant solutions were placed in a boiling water-bath for 2 min. in order to destroy histaminase and acetylcholine.

Histamine was assayed on the guinea-pig ileum, using an automatic assay apparatus (Boura, Mongar and Schild, 1954).

Calculation of Thermodynamic Activity

The activities of alcohols in aqueous solution were calculated from the activity coefficients given by

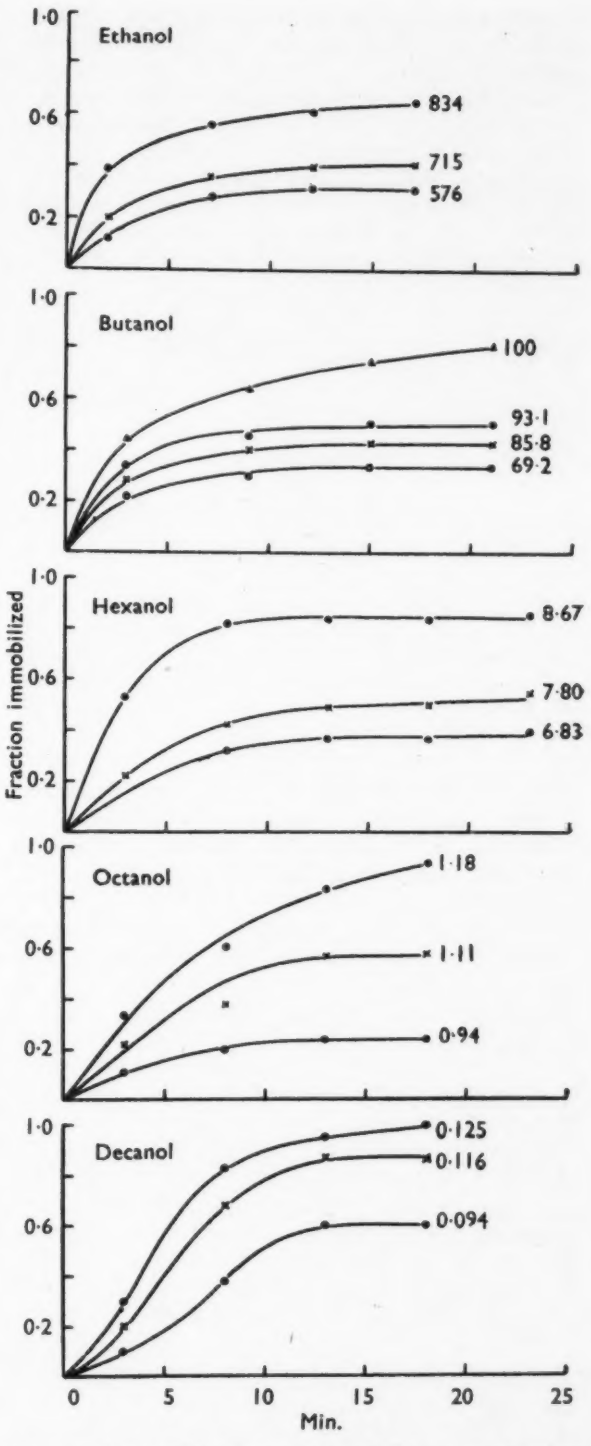

FIG. 1.—Time-action curves of alcohols on paramecium mobility. The figures appended to the curves show the alcohol concentration (mM). Each point represents the mean of three or four observations.

Butler, Thomson and MacLennan (1933), and the mole fraction of alcohol in solution, using the relation:

$$\text{Thermodynamic activity} = \text{Activity coefficient} \times \text{Mole fraction}$$

For alcohols dissolved in Tyrode solution, no measured activity coefficients are available and certain approximations were necessary. Lindenberg (1948) found that the activity of ketones in sodium chloride solution behaved according to the equation:

$\log (\gamma_s/\gamma_o)/\mu = K = cN$

where γ_s, γ_o = activity coefficients in salt solution and water respectively.

μ = ionic strength of salt solution.

K = displacement constant, peculiar to salt and organic solute.

c = proportionality constant, peculiar to organic solute.

N = molecular refraction of organic solute.

For ketones c = 6·3.

In the present measurements, this value of c was used and the ionic strength of Tyrode solution calculated to be 0.159. The correction was, in fact, small (10% for octanol, progressively less for lower alcohols).

Results

Paramecium Immobilization

The qualitative effects of alcohols on paramecium followed a fixed pattern. Paramecia normally move smoothly, rotating continuously about a longitudinal axis and occasionally changing direction abruptly. The first effect of alcohols is to cause their path to become spiral (coinciding with the rotation). The forward movement then becomes progressively slower and eventually stops. At this stage the anterior end is stationary while the posterior end moves in a circle. This movement gradually subsides.

These stages were clearer and the sequence took place more slowly with higher than with lower alcohols.

Such a sequence is unlikely to be the result of overall depression of the cell, and more probably represents a depression of ciliary action passing over the membrane in a fixed pattern.

Fig. 1 shows time-action curves of alcohols on paramecia for alternate members of the homologous series tested. In most cases a clear equilibrium was reached, the time taken for equilibration varying from about 8 min. with ethanol to about 13 min. with decanol. In a few cases (for instance, with the highest concentration of butanol and octanol) no clear equilibrium was established. Where this complication arose the percentage immobilization at the time of equilibrium with lower alcohol concentrations was used.

In other similar toxicity studies, various measures have been used as criteria of activity, such as percentage mortality after a standard time, time taken to kill 50%, etc. In view of the steadily increasing time taken for equilibration

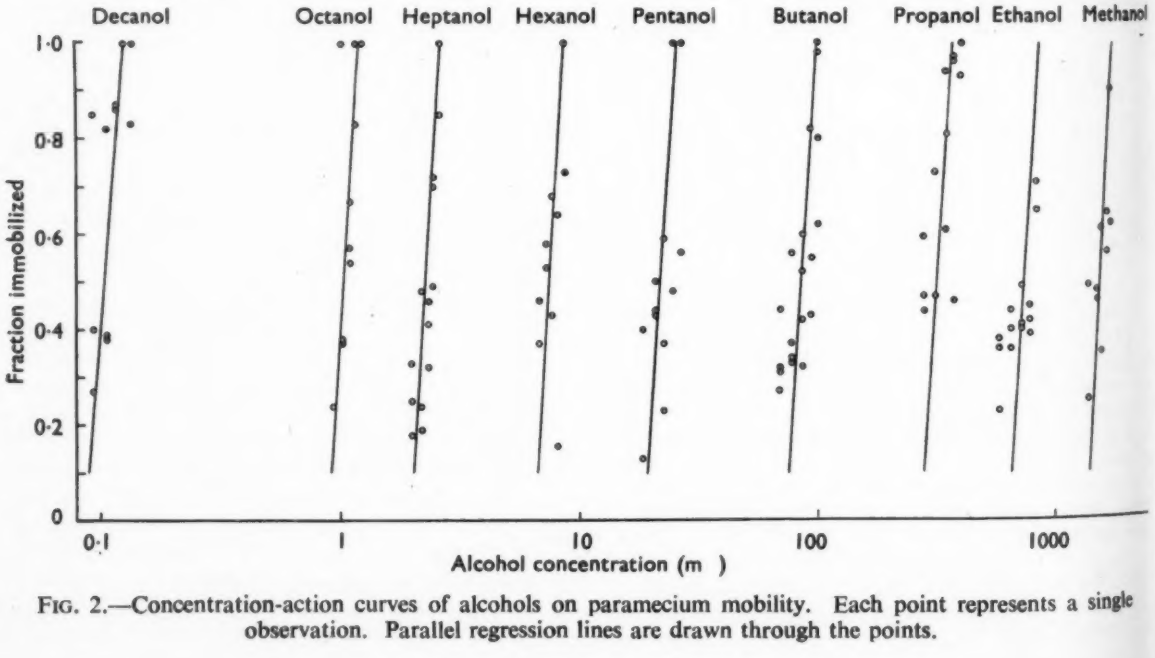

FIG. 2.—Concentration-action curves of alcohols on paramecium mobility. Each point represents a single observation. Parallel regression lines are drawn through the points.

observed in the homologous series of alcohols, it was felt that the criterion of activity must be independent of time relations. Hence percentage immobilization at equilibrium was used as a measure of pharmacological activity.

Fig. 2 shows the fraction of organisms immobilized at equilibrium plotted against alcohol concentration (on a logarithmic scale) for the series of alcohols from methanol to decanol (nonanol excluded). Each point represents a single determination. Since straight lines fit the points well the use of a probit transformation was considered unnecessary. The lines have all been plotted in accordance with a common regression coefficient, judged by eye. In no case does the regression line conflict with the trend of the points.

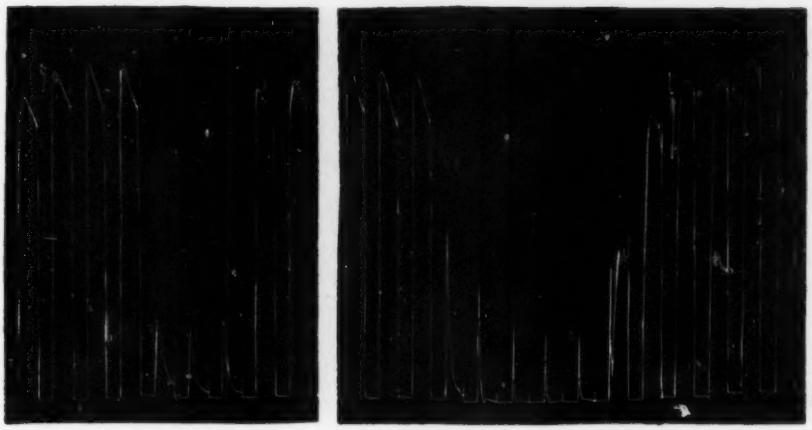

FIG. 4.—Effect of ethanol and octanol on maximal responses of the guinea-pig ileum to 1 μg./ml. acetylcholine.

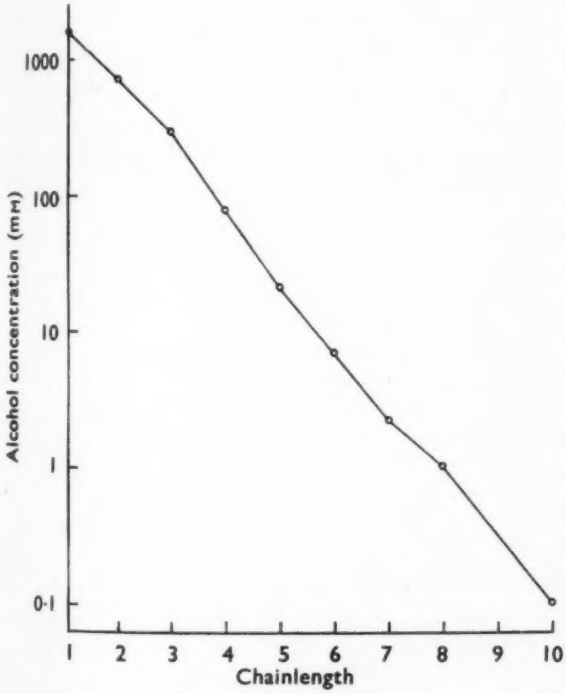

FIG. 3.—Relationship between alcohol chainlength (that is, the number of carbon atoms) and the concentration required to immobilize 50% of a population of paramecia. The concentrations are taken from the regression lines of Fig. 2.

Since these logarithmically-plotted regression lines are parallel, the ratio of concentrations of different alcohols required to immobilize a given fraction of organisms is independent of the fraction selected. Hence it is valid to use equiactive concentrations as a measure of the relative potencies of different alcohols.

The concentration of alcohol causing 50% immobilization at equilibrium has been used as a criterion for comparison. This concentration may be obtained from the regression lines in Fig. 2. The precision of these estimates may be expressed by the coefficient of variation, $\lambda(=0.039)$.

Fig. 3 shows the concentration of alcohol required for 50% immobilization plotted logarithmically against the number of carbon atoms in the chain. It shows a linear logarithmic decrease in equiactive concentration with increasing chainlength. Certain deviations from linearity are seen, namely a flattening at the top of the graph, and a flattening between heptanol and octanol. The flattening at the top has been found also in other experiments in the present series. The kink between heptanol and octanol, however, has not been observed elsewhere.

Depression of Gut Contractility

The action of alcohols is rapidly and completely reversible. This is shown in Fig. 4. The rate of recovery is somewhat less than the rate of development of the effect. If a series of equal doses of agonist are given, and then alcohol is added, the response declines in an exponential

FIG. 5.—Effect of ethanol on the responses of the guinea-pig ileum to acetylcholine. Acetylcholine concentration in μg./ml. is shown under each contraction.

manner, eventually flattening off to a steady lower value. Recovery after removal of alcohol is similarly exponential. The time required for equilibration with alcohols increased with chain-length from about 1 min. for methanol to about 5 min. for octanol. Decanol was found not to reach equilibrium after 20 min. exposure and recovery of the response after its removal was incomplete.

The specificity of the action of alcohols was tested by comparing their effects on contractions produced by histamine, acetylcholine, and electrical stimulation. In one experiment, equivactive doses of histamine and acetylcholine were given alternately, and alcohol was added. Alcohol affected both responses equally and simultaneously. In another experiment, maximal doses of histamine were alternated with maximal electrically-induced contractions (which were slightly smaller than the histamine contractions). Alcohol affected both these responses equally, the electrically-induced contractions remaining smaller than the histamine contractions by the same fraction. Hence the action of alcohols shows no specificity.

It was found that alcohols diminished both the gradient and the maximum of agonist log-concentration action curves. Fig. 4 shows that there is a qualitative modification of the contraction by alcohols. The normal response of smooth muscle to addition of acetylcholine is an immediate rapid contraction, followed by a slower phase which reaches a peak and is followed by gradual relaxation in the presence of acetylcholine. In the presence of a strongly depressant concentration of alcohol, relaxation sets in immediately after the rapid phase of contraction. There is no slow phase of contraction, and relaxation is often complete before the stimulant is washed out. This strongly suggests that alcohols interfere with the contractile mechanism itself rather than with the receptors. The simplest

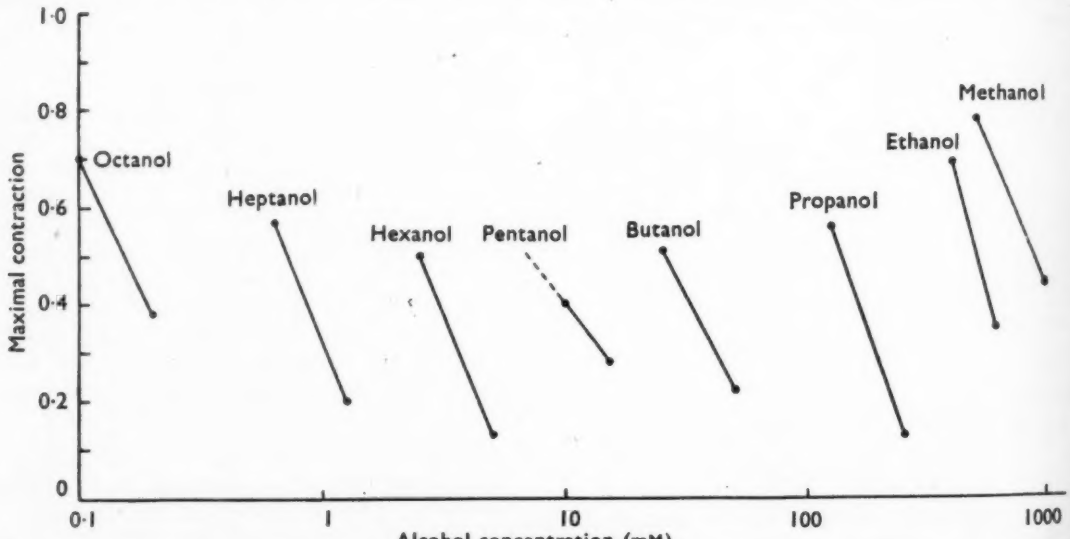

FIG. 6.—Concentration-action curves of alcohols on the responses of the guinea-pig ileum to acetylcholine. The ordinates represent the ratio of the maximal contractions in the presence and in the absence of alcohol. Each point represents a single determination and all were obtained on a single preparation.

way to measure such effects is by diminution of the maximal contraction (Arunlakshana and Schild, 1959).

In practice this was made difficult by the fact that, in the presence of depressants, the maximal contraction tends to diminish with successive doses of stimulant, as shown in Fig. 5. The acetylcholine dose was successively doubled in the presence of alcohol. The contractions increased in size up to a dose of 50 μg., but the response to the following dose of 100 μg. was considerably smaller. Increasing the dose interval reduced this effect, but considerably increased the error. The procedure adopted was to use a standard dose interval of just over one minute, and to take the largest response in a successively doubling series as the maximal response in the presence of depressant.

Preliminary experiments with alcohols showed that the relation between log-concentration of depressant and action in depressing the maximal contraction followed a sigmoid form, approximately linear between 20% and 80% effect, and tailing off symmetrically at either end. In the quantitative tests, therefore, each alcohol was applied in two concentrations, selected to give effects larger and smaller than 50% and to lie within the 20–80% range. The concentration required for a 50% effect, which was used as a criterion for comparison of potency, was obtained by linear interpolation.

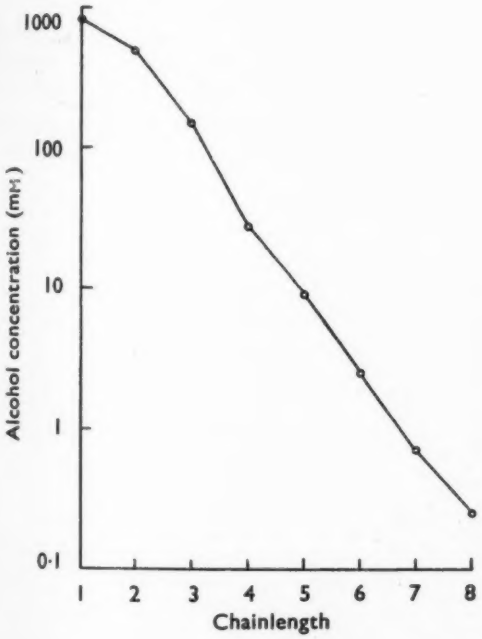

FIG. 7.—Relationship between alcohol chainlength and the concentration required to cause 50% depression of the maximal contraction of guinea-pig ileum to acetylcholine. The points plotted are the means of all the determinations.

Fig. 6 shows a series of two-point comparisons of the alcohols. Each point represents the mean of three or four observations on different ileum preparations. It was found that different preparations varied considerably in their sensitivity to alcohols, but the relative potencies of different alcohols varied much less. The coefficient of variation, λ, was 0.252, indicating an error about nine times that of the paramecium test. The parallel nature of the regression lines and their regular spacing in Fig. 6 is obvious. Fig. 7 shows the linear logarithmic decrease in the concentration required for 50% depression of maximal contraction (taken from the regression lines in Fig. 6) with chainlength. As observed with the paramecium immobilization experiments, there is a slight flattening between methanol and ethanol, but no other marked irregularity.

Depression of Lung Oxygen Consumption

Under the conditions of the experiment, oxygen uptake by lung particles in Tyrode solution continued at a steady rate for about 2½ hr. after placing the flasks in the Warburg apparatus. The rate of uptake in Tyrode solution, expressed as qO_2 (μl. absorbed per min. per mg. dry weight of tissue) varied from 2.5 to 5 in different animals, but samples from one animal gave consistent values.

In the presence of strongly depressant alcohol concentrations, the oxygen uptake frequently slowed and stopped after about 1½ hr. This was, however, always preceded by a period of steady uptake, from which the qO_2 was calculated. This cessation of oxygen uptake was probably due to the death of the tissue resulting from profound depression of its function.

As in the experiments on gut contractility, preliminary experiments showed that the log-concentration action curve was sigmoid in form, with a linear middle region, and two-point comparisons were again used. Fig. 8 shows the series of results obtained on the alcohol series. Each point represents a single experiment, and the straight lines in cases where more than two concentrations were used were drawn by eye. In the lower part of the series, from methanol to hexanol, the results are very similar to those obtained in the previous experiments in that the lines are all parallel and fairly regularly spaced,

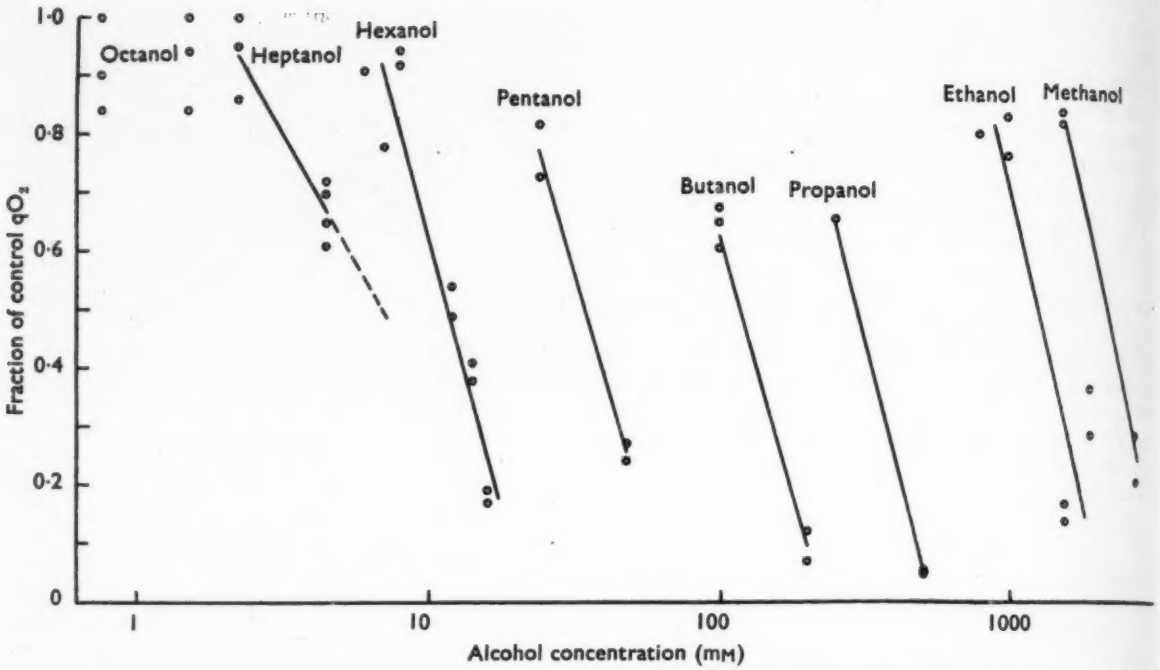

FIG. 8.—Concentration-action curves of alcohols on lung oxygen consumption. The ordinates represent the ratio of oxygen consumptions in the presence and in the absence of alcohol. Each point represents a single determination.

with the exception of methanol and ethanol. Above hexanol, however, the pattern changes completely. Heptanol shows a regression line less steep than the lower members, while octanol showed no significant activity at all. With both these alcohols the highest concentration used was close to saturation.

For the lower members of the series (hexanol downwards) the coefficient of variation, λ, was 0.182. Thus the variability was about six times that encountered in the paramecium tests, due partly to the lower gradient of the regression lines and partly to the greater scatter of the points.

The alcohol concentration required for 50% depression of oxygen consumption is shown plotted against chainlength in Fig. 9. This shows that, as far as hexanol, the pattern is the same as that found in the previous tests. The point for heptanol is taken from the extrapolated regression line of Fig. 8 and represents an unattainable concentration.

Throughout the series, the concentration required for 50% depression of oxygen consumption was approximately three times that required for 50% immobilization of paramecia and approximately 1.5 times that required for 50% depression of gut contractility.

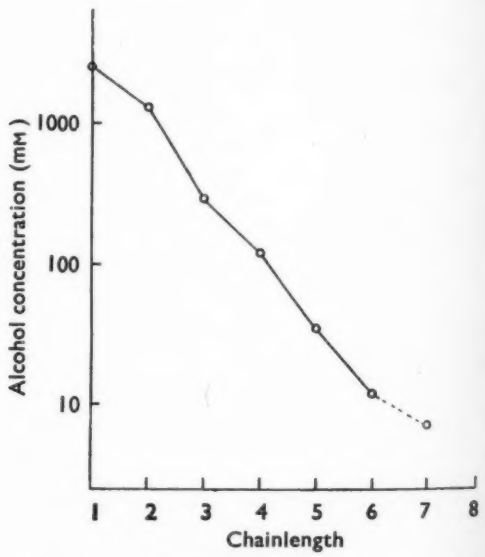

FIG. 9.—Relationship between alcohol chainlength and the concentration required to cause 50% depression of lung oxygen consumption. The value for heptanol was obtained by extrapolation of the regression line in Fig. 8, and represents an unattainable concentration.

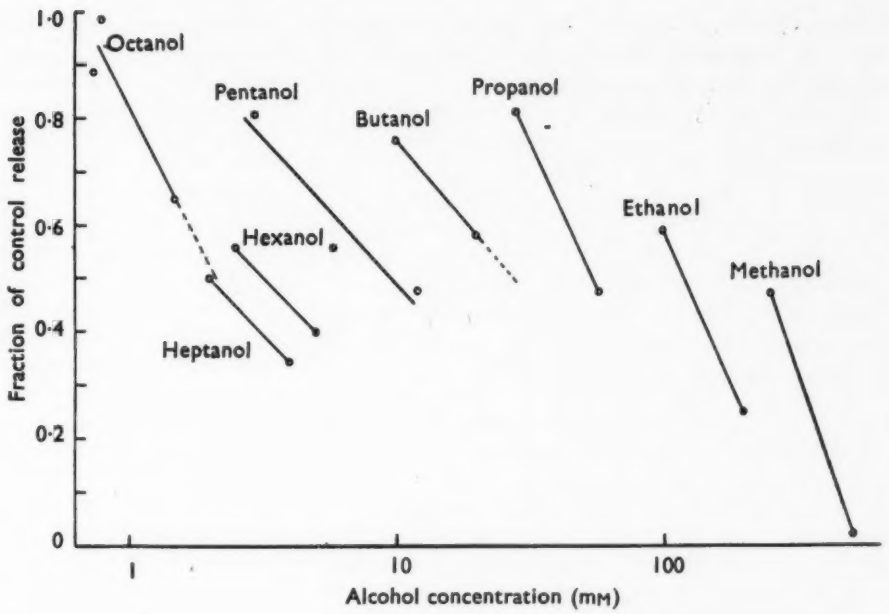

FIG. 10.—Concentration-action curves of alcohols on anaphylactic histamine release. Each point is the mean of all determinations at a particular alcohol concentration.

Depression of Lung Histamine Release

The amount of histamine released by antigen from chopped lung particles in the absence of any inhibitor varied considerably between preparations from different animals. Different samples from the same animal also showed some variation. The mean histamine release from fifty-three 0.2 ml. lung samples obtained from 18 animals was 0.96 μg. Expressed as a percentage of the total histamine, the variation was much less, and the mean value was 26%. In the absence of antigen under the same conditions the mean release was 3%.

As in the previous experiments, it was found that the log-concentration action curves for all these types of compounds were sigmoid in shape, with a middle linear region, and 2-point comparisons were again used.

The results obtained with the alcohol series are shown in Fig. 10. The deviations from parallelism are not significant. The coefficient of variation, λ, was 0.321, that is, the variability, was approximately ten times as great as that encountered in the paramecium experiments.

The spacing of the regression lines is fairly regular as far as hexanol, but the interval between hexanol and heptanol is less, and between heptanol and octanol there is no interval. The highest concentration of octanol used was close to saturation, and did not produce 50% inhibition. Thus, just as in the experiments on oxygen consumption, a cut-off point is reached, beyond which chainlength compounds show no activity. However, in the oxygen consumption experiments, heptanol showed a reduced gradient and an anomalously high equiactive concentration, and octanol showed no activity, while in these experiments both heptanol and octanol show the same gradient as the lower alcohols although their equiactive concentrations are anomalously high.

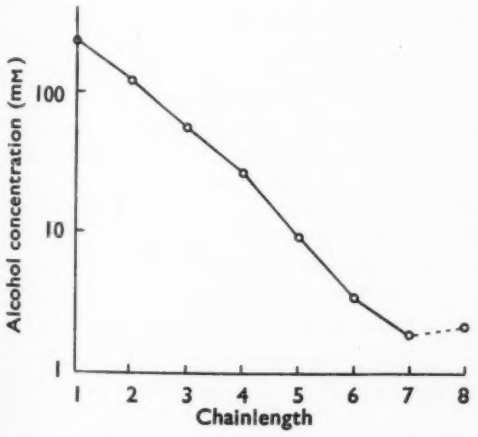

FIG. 11.—Relationship between alcohol chainlength and the concentration required to cause 50% depression of anaphylactic histamine release. The value plotted for octanol was obtained by extrapolation of the regression line in Fig. 10, and represents an unattainable concentration.

Fig. 11 shows alcohol concentration required for 50% inhibition of histamine release plotted against chainlength. Up to hexanol, the graph is approximately linear (no flattening between methanol and ethanol is apparent). The cut-off with octanol is clearly shown.

Discussion

The Log-Concentration Action Relationship

For each system investigated, a set of log-concentration action curves for the series of alcohols has been presented, and it is interesting to compare these figures. In each case the regression lines plotted were parallel over most of the range of alcohols investigated, but the gradient differs between different systems. Thus, in the tests on paramecium toxicity, the regression coefficient was 720, in the tests on gut contractility 90, in the tests on lung oxygen consumption 174, and in the tests on lung histamine release 59. In the paramecium toxicity test, the measurement was quantal, whereas in the other experiments it was almost certainly quantitative (one cannot be absolutely certain that these other effects were not due to the all-or-nothing depression of a certain proportion of cells, but this seems most unlikely). The coefficient given for the tests on paramecium toxicity is therefore not comparable with the other coefficients since it represents only the variability of the population, whereas the regression coefficient in quantitative experiments has other significance.

The constancy of gradient among different members of the homologous series indicates that the order of the reaction is constant. The difference in gradient between different biological systems may be interpreted in two ways. First, the reaction order may vary, which would suggest that the biophase shows fundamentally different properties in the different systems, and that the mode of action of alcohols in the different systems is different. In view of the characteristic pattern of activity in different members of the homologous series, it seems unlikely that the mode of action in different systems is as unrelated as this theory would suggest. Second, the discrepancies of gradient may be explained on the basis of a common mode of action if one supposes that the dependence of the different biological phenomena investigated on the single system which is affected by alcohols shows different quantitative relationships. Thus, the lung oxygen consumption (which has the steepest gradient) may show the complete range of inhibition, from 0 to 100%, over a narrow range of inhibition of the system which is the primary site of action of alcohols, whereas inhibition of histamine release takes place over a much wider range. This explanation appears to be more plausible than the first, since it preserves the unity of the action of unspecific depressants, which is the most striking feature evident in these results.

The differences which are observed in the higher and lower members of the homologous series are more difficult to explain than those of gradient between different systems. Methanol in most of the systems investigated showed a steeper gradient than the succeeding members of the series. It also showed an anomalous potency, greater than would be expected from its chainlength. Ferguson (1939) disposes of the anomalies of methanol by assuming that its physical toxicity is complicated by its chemical reactivity. This is a convenient explanation, but, without definite evidence that its mode of action differs from that of higher alcohols, it does not seem entirely justified. The fact that methanol shows similar anomalies in many of its physical properties (density, refractive index, etc.) does not dispose of the need to explain the anomalies in its biological action. Indeed, it would seem that if its biological distinctiveness could be explained in terms of its physical distinctiveness this would be a very useful step in the elucidation of the mechanism of action of unspecific depressants.

In the two systems investigated in which a cut-off in activity occurred in alcohols lower than octanol, an interesting discrepancy is evident. In the tests on depression of histamine release, no change in gradient of log-concentration action curves was evident before the cut-off activity. In contrast, the tests on depression of oxygen consumption showed that heptanol gave a regression line which was markedly less steep than that of the lower alcohols. Octanol showed no activity whatever. At present, no explanation is available to account for this.

Validity of the Concept of Unspecific Drug Action

The term " unspecific " implies that drugs of this class exert actions on a wide range of biological phenomena, and also that the relative potency of two different drugs is independent of the system on which they are tested. The investigations have verified the first implication, but the second is found not to hold. Fig. 12 is a composite diagram showing the effect of chainlength on equiactive alcohol concentration for the living

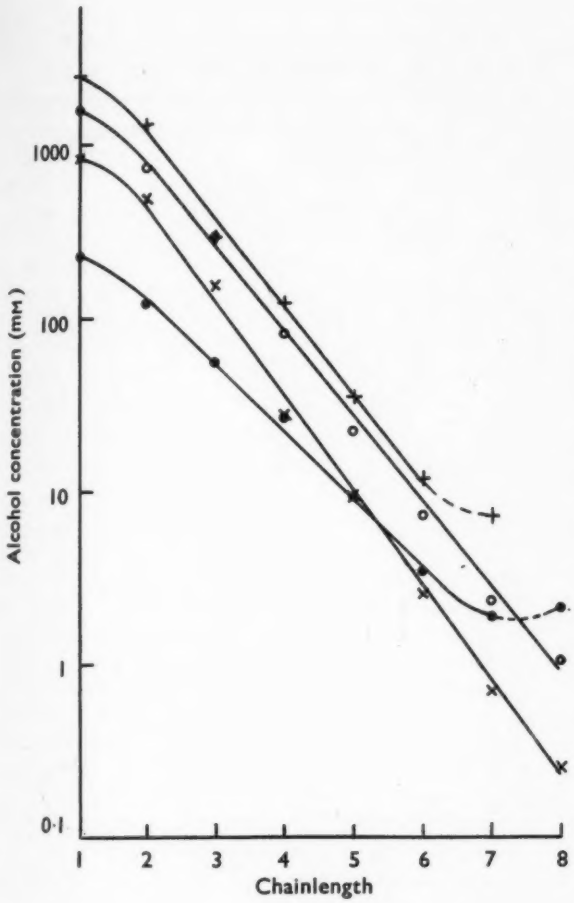

FIG. 12.—The relationship between alcohol chainlength and the concentration required for 50% depression of gut contractility (**X**), paramecium mobility (**O**), lung oxygen consumption (+), and lung histamine release (●). The curves have been smoothed out for clarity.

systems investigated. These lines are clearly not parallel. Thus equal concentrations of hexanol are required to cause 50% depression of both gut contractility and anaphylactic histamine release, while with ethanol the concentration required to depress gut contractility is about four times that required to inhibit histamine release. In this strict sense, therefore, the action is not truly unspecific, as ethanol might be said to be specific in depressing histamine release. Similarly, the phenomenon of cut-off means that alcohols completely lose their activity on one system, while retaining it on another. This again confers a type of specificity in certain higher alcohols. It seems, therefore, that the concept of completely unspecific drug action is untenable.

Ferguson's distinction of physical and chemical drug action seems to describe better the obvious differences between these classes of drugs. However, certain obstacles arise even with this distinction. There is the difficulty of deciding exactly what is meant by physical and chemical action. Ferguson and Hawkins (1949) have endeavoured to resolve this difficulty by investigating the toxic actions of very simple inorganic gases, including some inert gases, which are incapable of entering into chemical reactions. They found that these compounds exert the same actions as other narcotics acting by the alleged physical mechanism, and the thermodynamic activities required to produce a certain effect are in accordance with those found for other substances. Thus it is clear that no chemical process involving electron transfer takes place, but it would be difficult to demonstrate such a process taking place in the action of alleged chemically-acting drugs, whose action is entirely reversible. Indeed, it would seem quite possible that drugs acting at thermodynamic activities less than those compatible with Ferguson's criterion for physical activity might be doing so, not on account of any electron-transferring chemical process, but by virtue of some special molecular configuration which renders their physical interaction with the biophase much more disturbing than that produced by the wide range of undistinguished molecules which form Ferguson's physically-acting group.

We therefore have to turn to less direct criteria, of which the most illuminating is the thermodynamic activity of the compound required to produce an effect. Ferguson and Pirie (1948) found that the activities of compounds required to kill grain weevils fell into two clear groups. Those acting at activities between 0.1 and 1 they termed physical agents, and those acting at lower activities (usually very much lower) were termed chemical agents. Such a distinction separates the two groups very nicely if a single biological activity is considered, but different biological phenomena show quite wide variations in the thermodynamic activities of physically-acting drugs required to produce a given effect. Thus in the present investigation the thermodynamic activities of lower alcohols required to depress anaphylaxis were below 0.01, and in no case was an activity greater than 0.1 required with ethanol. This is obviously explained by the smaller disturbance of function which was used as a

TABLE I

CONCENTRATIONS (mM) AND THERMODYNAMIC ACTIVITIES OF ALCOHOLS REQUIRED FOR 50% DEPRESSION OF FUNCTION IN THE BIOLOGICAL SYSTEMS INVESTIGATED

Alcohol	Paramecium Mobility		Gut Contractility		Lung Oxygen Consumption		Lung Histamine Release	
	Concentration	Thermodynamic Activity	Concentration	Thermodynamic Activity	Concentration	Thermodynamic Activity	Concentration	Thermodynamic Activity
Methanol	1,600	0·0466	821	0·023	2,500	0·071	230	0·0065
Ethanol	730	0·0588	476	0·032	1,320	0·085	120	0·0077
Propanol	300	0·0694	149	0·035	298	0·070	56	0·0132
Butanol	81	0·0838	26·6	0·024	121	0·117	26·5	0·0234
Pentanol	21·8	0·105	9·0	0·038	34·4	0·145	9·5	0·0400
Hexanol	7·6	0·150	2·5	0·044	11·9	0·208	3·5	0·0613
Heptanol	2·3	0·178	0·71	0·049	7·1	0·497	1·9	0·133
Octanol	1·05	0·279	0·25	0·061			2·2	0·537

criterion of potency. In Ferguson and Pirie's investigation, the response observed was death.

Gavaudon, Dodé, and Poussel (1944), investigating the thermodynamic activities of compounds required to narcotize aquatic animals, found, similarly to Ferguson, that the activities required fell into two groups, but they suggested that the upper limit of activity for a chemically-acting compound was about 0.04. Clearly this limit cannot be set at any fixed value, since it will depend on the system investigated. The distinction between the activities of the two types of drug on any one system is, however, a useful criterion, and is good evidence that separate mechanisms of action are involved.

Relation Between Chainlength and Thermodynamic Toxicity

The term "thermodynamic toxicity" will be used to denote the inverse of the thermodynamic activity of a compound required to produce a certain effect. No theoretical significance attaches to the phrase.

Ferguson (1939) found, in general, a decrease of thermodynamic toxicity with chainlength within a homologous series, but Brink and Posternak (1948) found that it was constant. This is a question of some theoretical interest, and one which should have a definite answer.

Table I shows the thermodynamic activities of alcohols required for 50% depression of function in the four living systems investigated, and Fig. 13

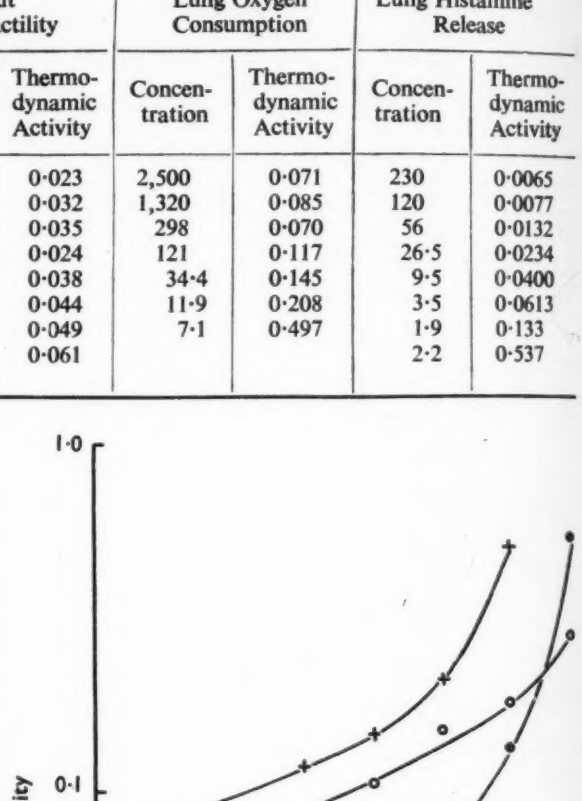

FIG. 13.—Relationship between alcohol chainlength and the thermodynamic activity required for 50% depression of gut contractility (X), paramecium mobility (O), lung oxygen consumption (+), and lung histamine release (●).

shows this plotted logarithmically against chain-length.

In the curves in Fig. 13, it is noticeable that the two cases in which no cut-off occurred, namely, in the tests on paramecium and gut contractility, the curves approximate to a straight line (a large deviation at butanol is evident in the gut contractility curve; this is probably error), while the other two curves in which cut-off was observed show a marked steepening at the top. It must be remembered that both of those tests were carried out in Tyrode solution, and are subject to approximations in the calculation of thermodynamic activity. It is not felt, however, that this can account entirely for the difference.

According to Ferguson's hypothesis, cut-off results from a steady decrease in thermodynamic toxicity with chainlength. Thus at a certain chainlength the required thermodynamic activity exceeds unity and is unattainable. According to this view, there is no reason to anticipate a sharp increase in the required thermodynamic activity immediately preceding the cut-off. However, the present results show that just before the cut-off the thermodynamic activity required is greater than would be predicted from the trend of the thermodynamic toxicity of preceding members of the series. Hence these results agree with Ferguson in that they show a decrease of thermodynamic toxicity with chainlength, and with Brink and Posternak in that they indicate a more rapid decrease of thermodynamic toxicity immediately preceding the cut-off. This conclusion is in agreement with most of the previous quantitative investigations of homologous series, in which it is found that complete cut-off is preceded by reduced thermodynamic toxicity.

It is thus necessary to explain not only the steady fall in thermodynamic toxicity with chainlength but also the more rapid fall which precedes the cut-off. At present no explanation is available for either of these observations, and an explanation will probably be found only when it is possible to test a concrete hypothesis of the mechanism of action of physically-acting drugs by comparing predictions with experimental results.

One feature of the results of Clark (1930) on heart muscle which defies explanation by Ferguson's hypothesis is that in some experiments the higher alcohols were applied in a concentration exceeding that of a saturated solution, in the form of what Clark calls a "colloidal suspension," obtained by prolonged shaking. Clark found that the concentration-action relationship showed no anomaly when such suspensions were used, yet their thermodynamic activity cannot exceed that of a saturated solution.

Mechanism of Action

Mullins (1954) suggested the cell membrane as the site of action, envisaging that the interstices in the lattice structure, which normally confer properties of permeability on it, become blocked up by inert foreign molecules, and the resulting decrease in permeability is responsible for the depression of function. It is indeed difficult to formulate any other mechanism by which the mere presence of inert molecules in a cell can depress its function, and in spite of the inadequate experimental proof of this hypothesis (see appendix) it seems the most probable explanation. It is perhaps an over-simplification to assume that the external cell membrane is the seat of this action: it seems just as likely that some internal barrier, such as the membrane enveloping the mitochondria or other cytoplasmic inclusions, is the site of action, or possibly many different membranes may be involved.

It seems likely that physically-acting depressants in sufficient concentration will depress any aspect of cell function. The question thus arises whether all the functions are depressed in turn as a result of progressive depression of one single system (possibly an enzyme system or decreased permeability of the outer cell membrane), or whether they are depressed individually by these drugs. This question cannot at present be answered. Experiments on the action of unspecific depressants on viable sub-units of the cell, such as mitochondria, might prove illuminating. If it were found that mitochondria were depressed by alcohols in the same way as whole cells, this would be evidence of independent depression of the different units of a living cell, but, if the action on mitochondria differed from that characteristic action on whole cells, then one might assume that depression of some single unit of the cell was responsible for the secondary depression of its many different functions.

Another line of research which might possibly prove constructive in elucidating the mechanism of action is the investigation of the action of alcohols on some synthetic non-living membrane. Thus it would probably prove possible to produce a monolayer of orientated protein molecules, and to study its permeability and the effect of alcohols on it. In this way it might be possible to test Mullins' theory on a system in which it is known that there are no enzymes, and whose physical chemistry should be much easier to work out than that of a cell membrane.

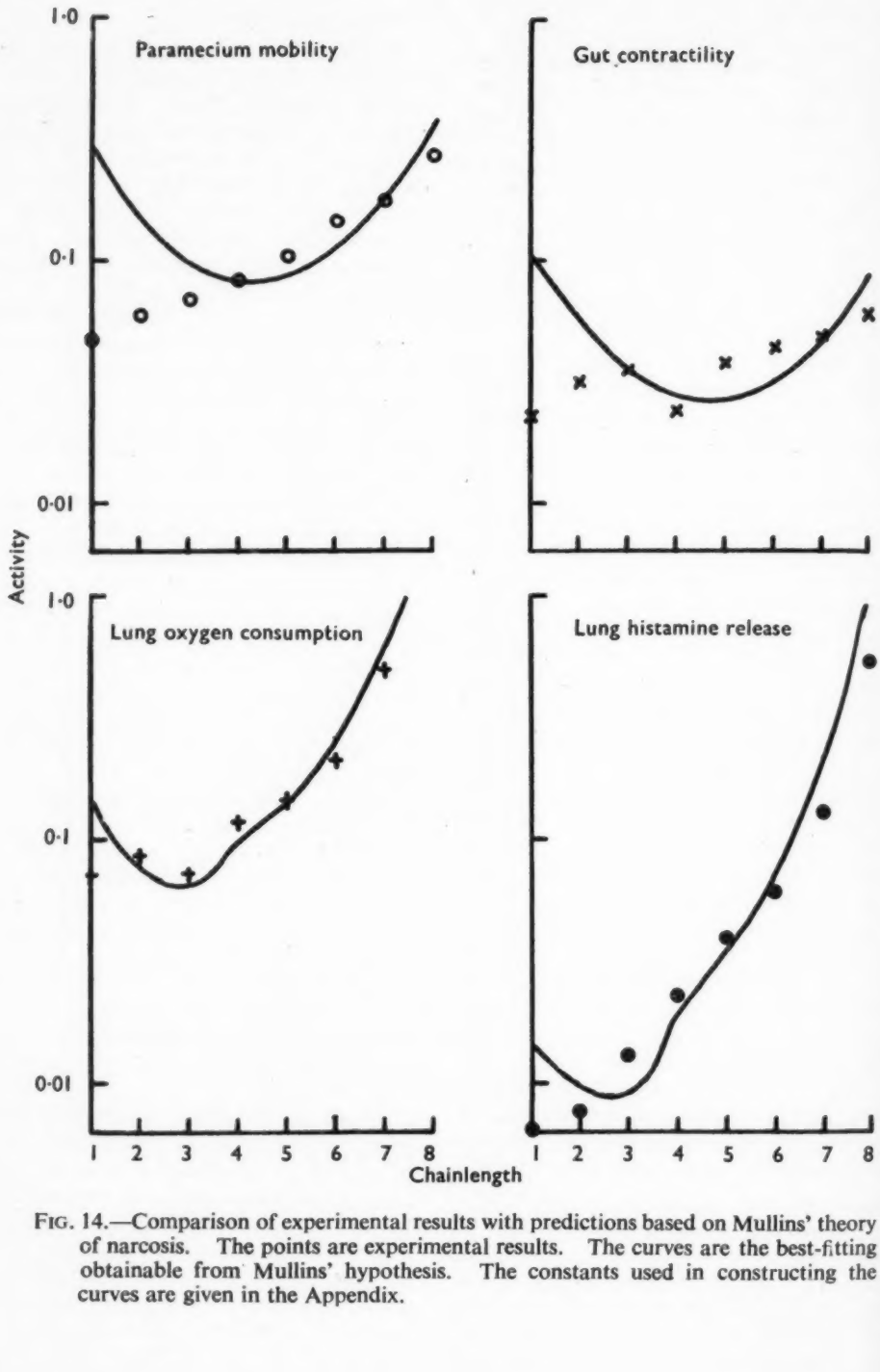

FIG. 14.—Comparison of experimental results with predictions based on Mullins' theory of narcosis. The points are experimental results. The curves are the best-fitting obtainable from Mullins' hypothesis. The constants used in constructing the curves are given in the Appendix.

APPENDIX

Application of Mullins' Hypothesis

Mullins (1954) suggested that narcotics act by dissolving in some phase within the cell (probably a membrane) and that equal narcotic action occurs when an equal volume fraction of the membrane is occupied by narcotic molecules, regardless of their chemical structure.

In order to test this hypothesis, it is necessary to estimate the concentration of narcotic in the biophase. Mullins attacked this problem by estimating the activity coefficient of narcotic dissolved in the biophase, using principles put forward by Hildebrand and Scott (1950). He thus derived the following relationship:

$$\ln\gamma_{nar} = \frac{V_m(\delta_{nar}-\delta_{mem})^2}{RT} \quad \ldots\ldots\ldots\ldots(1)$$

where γ_{nar} = activity coefficient of narcotic in membrane.

V_m = molar volume of narcotic.

δ_{nar}, δ_{mem} = solubility parameter of narcotic and membrane respectively.

This equation is based on theories applying to mixtures of non-polar solvents. A large error may be incurred by applying it in the present circumstances.

The volume fraction of narcotic in the membrane is given by the expression, $X_{nar}V_m$, where X_{nar} is the mole fraction of narcotic in the membrane.

$\gamma_{nar}X_{nar}$ = A_{nar}, the thermodynamic activity required for narcosis.

$\therefore A_{nar}V_m = \gamma_{nar}X_{nar}V_m$.

According to Mullins' hypothesis, $X_{nar}V_m$ is constant.

$\therefore A_{nar}V_m \propto \gamma_{nar}$ .....(2)

In order to calculate γ_{nar} from equation (1), δ_{mem} must be known. This cannot be measured directly. A series of curves of log γ_{nar} against chainlength for different values of δ_{mem} were plotted according to equation (1) using values of V_m and δ_{nar} given by Mullins. According to Mullins' hypothesis (equation 2), one of these curves should be the same shape as the experimental plot of log $A_{nar}V_m$ against chainlength. Thus from each of the four biological systems investigated a value of δ_{mem} giving the best fit between experimental data and theoretical prediction was selected. Fig. 14 shows the experimental data (points) compared with the best fitting theoretical curves. The values of δ_{mem} and $X_{nar}V_m$ used in fitting the curves are as follows:

	$X_{nar}V_m$	δ_{mem}
Paramecium mobility	7·34	11·8
Gut contractility	2·52	11·5
Lung oxygen consumption ..	5·25	12·9
Lung histamine release ..	0·63	13·5

With the paramecium and gut contractility experiments the fit is very poor, but it is much better in the experiments on histamine release and oxygen consumption. However, the striking feature of the theoretical predictions—the minimum at a certain chainlength—is entirely lacking in the experimental results. Some of Mullins' results, relating to depression of nervous tissue, do in fact show this minimum, and the fit is much more convincing. Mullins found that the membrane solubility parameter giving the best fit was about 11, while in the present case it was about 13 in the only cases in which a possible fit was obtained. A parameter of 11 corresponds to a molecule with approximately the same ratio of hydrophilic and hydrophobic groups as butanol, which corresponds roughly with what is known of the cell membrane. A parameter of 13 corresponds to the hydrophilic/hydrophobic ratio of ethanol, which is most unlikely to correspond with the cell membrane.

The present results do not, therefore, support Mullins' hypothesis. However, the theoretical predictions are based on doubtful assumptions, and allow no firm conclusions to be drawn.

My sincere thanks are due to Dr. H. O. Schild, whose advice and encouragement were invaluable.

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THE ACTION OF LOCAL ANAESTHETICS ON THE SPINAL CORD OF THE CAT

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(RECEIVED DECEMBER 12, 1959)

The effects of intravenously injected lignocaine, procaine and amethocaine on the monosynaptic and polysynaptic reflexes and the cord dorsum potentials elicited by selective electrical stimulation of appropriate afferent fibres have been investigated in decerebrate cats before and after strychnine. The three drugs depress the amplitude of the reflex responses in the basal preparation for 40 to 50 min. Smaller doses eliminate the reflex augmentation produced by strychnine for comparable periods. Very small doses selectively abolish the augmentation of the P component of the cord dorsum response induced by strychnine. The drugs have no effect on post-tetanic potentiation of the monosynaptic reflex response evoked from the gastrocnemius muscle in the cat. Their activity on the spinal cord is roughly proportional to their local anaesthetic potency. The ratios of the doses which produce the same central activity are amethocaine 3: lignocaine 10: procaine 20.

It is known that, in addition to their peripheral effects, local anaesthetics when injected intravenously can modify the activity of the central nervous system in animals and man. Bernhard and Bohm (1954, 1955) showed that intravenous injections of local anaesthetics shortened or abolished the cortical after-discharge evoked by electrical stimulation of the cerebral cortex in monkeys. Bernhard, Bohm, Höjeberg, and Melin (1956) found that intravenous lignocaine would prevent the electroencephalographic appearance of cortical seizure activity on photic stimulation in epileptic patients. Evidence was presented by Bernhard, Bohm, and Höjeberg (1955) which suggested that intravenous lignocaine would prevent or stop both focal and generalized epilepsy, and Taverner and Bain (1958), using a "double-blind" trial technique, showed that this drug was an effective anticonvulsant in patients with status epilepticus.

In both animals and humans Bernhard *et al.* (1954, 1955) observed that local anaesthetics had little effect on the normal activity of the central nervous system, in contrast to their striking effect upon over-active neurones. The present investigation was undertaken to study the effect of local anaesthetics on the spinal cord of the cat under basal experimental conditions and after over-activity had been induced. The results show that intravenous local anaesthetics have effects on the spinal cord in cats which are comparable to those they exert on the brain, and that the central activity of the drugs tested is proportional to their local anaesthetic potency.

METHODS

The experiments were performed upon cats. Decerebration or spinal section was carried out under ether anaesthesia 2 to 3 hr. before the experiments began. The details of the electro-physiological technique have been reported (Bernhard and Taverner, 1951; Taverner, 1952, 1954). The rate of stimulation was once every 4 sec. The height of the main component of the polysynaptic reflex response was measured in preference to measurement of its total area. Previous personal studies by the present author, confirmed in the present work, have shown that this method provides an acceptable, though slightly less accurate, measure of the drug induced changes in a particular polysynaptic reflex evoked under standard conditions of stimulation and recording. The drugs used were amethocaine hydrochloride (0.1%), tubocurarine chloride (0.1%), lignocaine hydrochloride (0.1%), procaine hydrochloride (0.1%), and strychnine hydrochloride (0.01%), and amounts given in the text refer to weights of the salts in each case. They were injected slowly through a polythene tube lying in the brachial vein. The arterial blood pressure was recorded from the common carotid artery.

RESULTS

Electrical stimulation of the group I low-threshold afferent fibres in the nerve to the

gastrocnemius muscle is followed after an interval of 2.5 to 3 msec. by a synchronous reflex discharge in the S1 ventral root. It is usually accepted that this discharge represents activity in two-neuronal monosynaptic reflex arcs, and the efferent nerve impulses run in fibres which pass to the muscles from which the afferent fibres arise (Lorente de Nó, 1935; Renshaw, 1940; Lloyd, 1943). This electrical activity is probably equivalent to the myotatic reflexes or tendon jerks of clinical medicine. Electrical stimulation of a cutaneous nerve such as the sural results in an asynchronous, irregular discharge which appears in the S1 ventral root about 4 to 5 msec. later and lasts for 10 msec. or more. Such impulses have been shown by Lloyd (1943) to represent activity in more complex reflex pathways with several interpolated, internuncial neurones (polysynaptic reflexes). It is believed that the flexor and crossed extensor reflexes depend upon such polysynaptic chains.

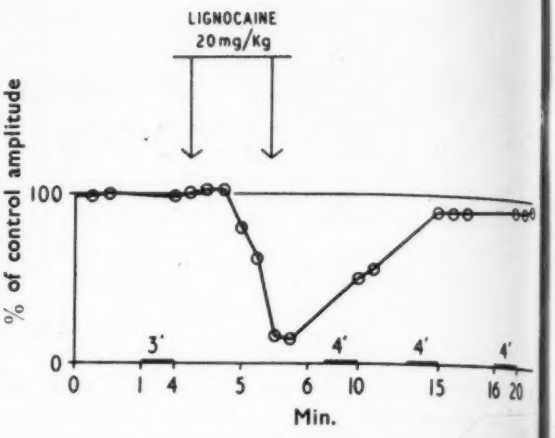

FIG. 1.—Effect of 20 mg./kg. lignocaine on the monosynaptic reflex evoked by stimulation of the nerve to the gastrocnemius muscle. Decerebrate cat. Ordinate: Reflex response as percentage of pre-injection amplitude. Abscissa: Time in min. Bars on base line represent suspension of recording.

Action of Local Anaesthetics on the Spinal Reflexes in the Decerebrate Preparation

Lignocaine.—The intravenous injection of doses of less than 5 mg./kg. lignocaine had no effect on the spinal reflexes. In about half the preparations 5 mg./kg. lignocaine intravenously caused a reduction in amplitude of the polysynaptic reflex to 50% of its pre-injection level, but the monosynaptic reflex was only reduced by about 20% of its pre-injection level. These reductions in amplitude were transient and the reflexes had returned to their pre-injection level within 20 min. Larger doses of lignocaine gave greater and more persistent reductions in the amplitude of the two reflexes.

Fig. 1 shows the effect of injecting 20 mg./kg. lignocaine intravenously in a decerebrate preparation. The injection was given slowly and no significant change in the blood pressure was observed. 30 sec. after the start of the injection the amplitude of the monosynaptic reflex began to fall, and by the end of the injection it was only 15% of the pre-injection amplitude. The reflex was tested at intervals, and it was found that recovery began about 5 min. after the injection and was complete within 10 min. The effect of the same dose of lignocaine (20 mg./kg.) on the polysynaptic reflex is shown in Fig. 2. The reflex began to fall in amplitude soon after the beginning of the injection, and had fallen to 10% of the pre-injection level before the injection was finished. Recovery began about 10 min. later but was slow. 20 min. later the reflex was still only 50% of the pre-injection amplitude and it did not recover until almost 60 min. after the injection. Doses of lignocaine larger than 20 mg./kg. were not given.

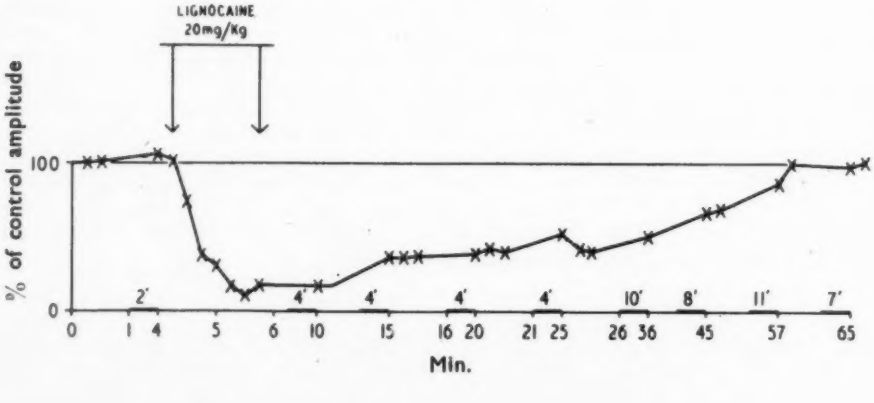

FIG. 2.—Effect of 20 mg./kg. lignocaine on the polysynaptic reflex evoked by stimulation of the sural nerve. Details as Fig. 1.

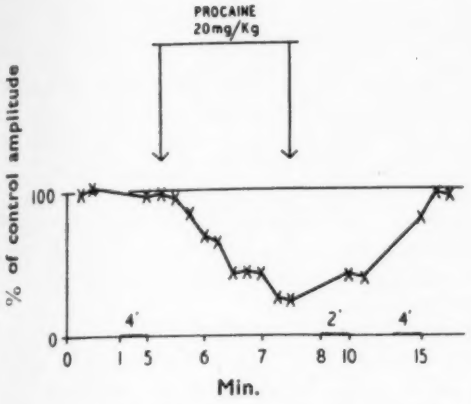

FIG. 3.—Effect of 20 mg./kg. procaine on the polysynaptic reflex evoked by stimulating the sural nerve. Details as Fig. 1.

Procaine.—Intravenous procaine had similar but much less marked effects on these reflexes. Fig. 3 shows the effect of 20 mg./kg. procaine on the polysynaptic reflex in a decerebrate preparation. The amplitude of the reflex fell to 25% of the pre-injection level at the end of the injection but had returned to normal within 15 min. There was no definite effect on the monosynaptic reflex with this dose of procaine. Larger doses of procaine were not given.

Amethocaine.—Amethocaine was found to have effects similar to those of lignocaine but at much lower dose levels. Fig. 4 shows the effect of the injection of 3 mg./kg. amethocaine on the spinal reflexes of a decerebrate preparation. The monosynaptic reflex fell to 50% of the pre-injection level and began to recover about 15 min. later. The reflex had returned to its pre-injection amplitude after 25 min. Similar but more marked changes were observed in the amplitude of the polysynaptic reflex after the same dose of amethocaine. The amplitude fell to 30% of the pre-injection level, recovery began after about 15 min. and the reflex had returned to its pre-injection amplitude 30 min. after the injection. Larger doses of amethocaine produced more profound and longer lasting changes in both reflexes, but with larger doses there was difficulty in maintaining the blood pressure in the preparations. In all experiments it was found that the polysynaptic reflex was affected more than the monosynaptic reflex by intravenous local anaesthetics and that both the extent of the depression and its duration were related to the dose given. Amethocaine was approximately 4 to 5 times as active as lignocaine in its effect on the spinal reflexes.

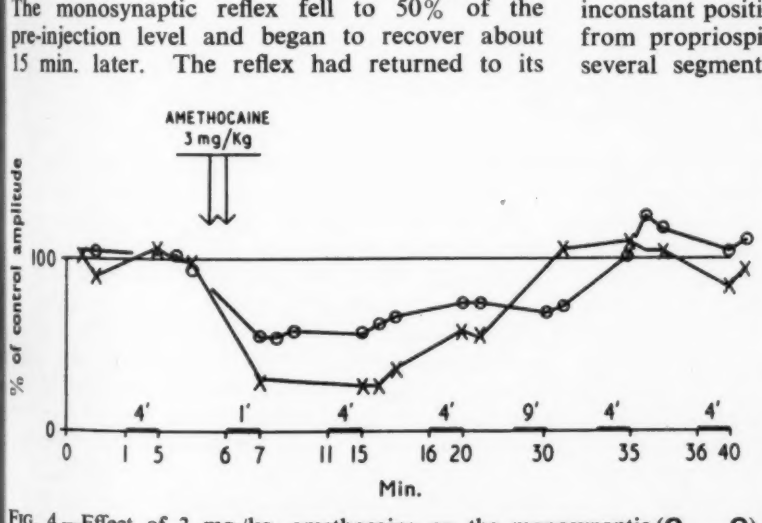

FIG. 4.—Effect of 3 mg./kg. amethocaine on the monosynaptic (O——O) and polysynaptic reflexes (X——X). Details as Fig. 1.

Action of Intravenous Local Anaesthetics on Strychninized Decerebrate Preparations

Strychnine causes a marked increase in the amplitude of the polysynaptic reflex and an increase in the P wave of the cord dorsum potential evoked by stimulating a cutaneous nerve such as the sural. In these experiments the cord dorsum potentials were recorded by a monopolar electrode placed on the dorsal surface of the first sacral spinal segment. It has been suggested by Bernhard and Widén (1953) that the N1 potential represents activity in monosynaptically activated cell bodies in the dorsal grey matter which do not participate in reflex transmission. The N1 deflection is followed by an inconstant positivity (P deflexion) which originates from propriospinal interneurones extending over several segments of the spinal cord. Taverner (1952) showed that strychnine increased the amplitude of the P deflexion in all types of preparation and increased the amplitude of the N1 deflexion in preparations with low spinal cord section.

Action on Polysynaptic Reflex Responses.—It was found that intravenously injected local anaesthetics were able to reverse the effect of strychnine in much smaller doses than those which modified the activity of preparations which had not received strychnine. Fig. *5a* shows the changes in amplitude of the

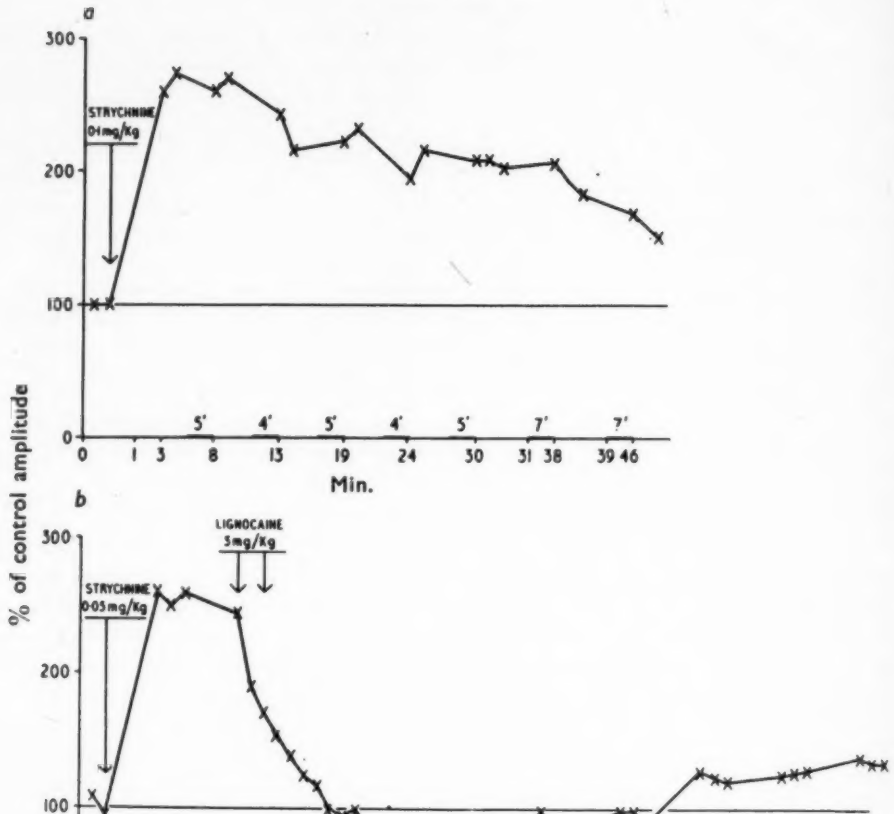

FIG. 5.—*a*, Effect of 0.1 mg./kg. strychnine hydrochloride on the polysynaptic reflex evoked by stimulation of the sural nerve. *b*, Same preparation. Effect of 5 mg./kg. lignocaine on the polysynaptic reflex after the injection of a further 0.05 mg./kg. strychnine hydrochloride. Details as Fig. 1.

polysynaptic reflex immediately after the injection of 0.1 mg./kg. strychnine hydrochloride intravenously in a curarized decerebrate preparation. The reflex increased in amplitude to about 250% of the pre-injection level and then declined slowly in amplitude but did not reach the pre-injection level 60 min. after the injection. Fig. 5*b* shows the effect of a further injection of 0.05 mg./kg. strychnine hydrochloride in the same preparation. The polysynaptic reflex again increased to 250% of the pre-injection level and the same gradual fall in amplitude began. 7 min. after the strychnine was injected, 5 mg./kg. lignocaine was injected intravenously. There was an immediate fall in the amplitude of the polysynaptic reflex to below the pre-strychnine level. 3 min. later the reflex was 75% of the pre-strychnine level, and 20 min. later it had only reached its normal pre-strychnine level. 30 min. after the injection the polysynaptic reflex had begun to increase in amplitude again and was then about 130% of its pre-strychnine value. It remained at this level for a further 30 min., but eventually fell to the pre-strychnine level again.

Similar results were obtained after the injection of procaine in doses of more than 10 mg./kg. but doses of less than 5 mg./kg. had no effect. 2 mg./kg. amethocaine gave comparable reduc-

tions in the amplitude of the polysynaptic reflex after augmentation by strychnine.

Action on Cord Dorsum Potentials.—The action of lignocaine on the cord dorsum potentials is illustrated in Fig. 6. The injection of 1.3 mg./kg. strychnine in a decerebrate preparation with low spinal section at the junction of the dorsal and lumbar segments of the spinal cord caused an increase in the amplitude of the N1 potential to 130% of the pre-injection level. At the same time a P deflexion appeared and its maximum amplitude has been taken as 100%. These changes persisted for 20 min. when an injection of 1 mg./kg. lignocaine was given. The N1 potential was unchanged, but there was an immediate fall in the amplitude of the P deflexion, which rapidly disappeared and did not return.

Similar results in other preparations were obtained with 0.4 mg./kg. amethocaine and with 2 mg./kg. procaine.

Action of Local Anaesthetics on Post-tetanic Potentiation

The amplitude of a monosynaptic reflex response can be increased two- or three-fold by tetanic stimulation of its afferent pathway (Lloyd, 1949). This effect persists for 1 to 2 min., and it has been suggested that it results from temporary physical enlargement of the synaptic endings of the afferent nerve fibres (Eccles, 1953). Tetanic stimulation of polysynaptic reflex pathways has little effect (Lloyd, 1949).

Post-tetanic potentiation of the monosynaptic reflex response evoked by stimulation of the nerve supplying one head of the gastrocnemius muscle was studied before and after the intravenous injection of the three local anaesthetics. Doses comparable to those producing changes in normal spinal cord reflex activity were shown not to have any significant effect on the degree or duration of the post-tetanic potentiation of the monosynaptic reflex response.

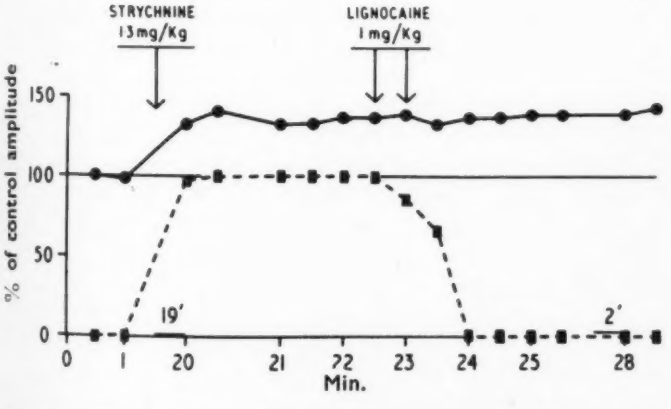

FIG. 6.—Effect of 1.3 mg./kg. strychnine hydrochloride followed by 1 mg./kg. lignocaine on the N1 (●——●) and P (■— — —■) components of the cord dorsum potentials evoked by stimulating the sural nerve. Details as Fig. 1.

DISCUSSION

In this investigation the observations of Bernhard and Bohm (1954, 1955) on the actions of systemic local anaesthetics on the cerebral cortex of the monkey have been extended to the spinal cord of the cat. The reduction in spinal cord activity after the injection of these drugs is of the same order and duration as that found in the brain. The mechanism of this effect is unknown.

It was found that much smaller doses of these agents were required to reverse the effects of strychnine on the spinal cord reflexes and on the cord dorsum potentials than were necessary to depress them in the absence of strychnine. The duration of action, however, was generally the same. In spite of the similarity of this action to the abolition by local anaesthetics of cortical over-activity after electrical stimulation and in clinical epilepsy, it is doubtful if the two forms of over-activity are comparable. There is now strong evidence that the central excitatory action of strychnine is due to the selective depression of both direct and indirect inhibition, and the mechanism may be one of competition with the inhibitory transmitter for the steric configurations on the inhibitory post-synaptic membrane (Bradley, Easton, and Eccles, 1953). The failure of local anaesthetics to influence the amplitude and duration of the post-tetanic accentuation of the monosynaptic reflex response is perhaps suggestive that local anaesthetics exert their action upon the inhibitory post-synaptic membrane, but there is no direct evidence at present.

The relative potency of the three drugs studied was found to be related to their local anaesthetic activity. There was some variation in different experiments, but in general amethocaine was more effective than lignocaine, which was itself more potent than procaine. 3 mg./kg. amethocaine intravenously produced the same reduction in amplitude of evoked

spinal cord activity as 10 mg./kg. lignocaine and 20 mg./kg. procaine. A similar relationship holds for the activity of these drugs in preparations which had received strychnine. These proportions correspond well with the relative doses of the three drugs needed to produce the same local anaesthetic effect, which are approximately 1 : 5 : 10 (Goodman and Gilman, 1955).

I am indebted to the Medical Research Council for a grant towards the expenses of this investigation. I am grateful to Professor A. Hemingway for the generous provision of laboratory facilities.

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Brit. J. Pharmacol. (1960), **15**, 207.

THE GANGLION-BLOCKING ACTIVITY OF AMINOBICYCLO-[2,2,1]HEPTANES (CONGENERS OF MECAMYLAMINE) AND BICYCLO[3,2,1]AZAOCTANES (BRIDGED CONGENERS OF PEMPIDINE)

BY

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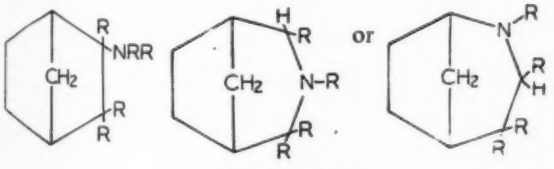
The structural requirements for strong ganglion-blocking activity and long duration of action amongst some lower homologues of mecamylamine, together with the discovery of similar activities amongst isomers with enlarged ring structures, are described. In both series of compounds it was found that the successive introduction of *C*-methyl groups surrounding the nitrogen atom resulted in a progressive increase in ganglion-blocking activity and duration of action.

The pharmacology of the potent ganglion-blocking compound pempidine, 1,2,2,6,6-pentamethylpiperidine, has recently been described (Corne and Edge, 1958; Spinks, Young, Farrington, and Dunlop, 1958).

The independent discovery of the properties of this compound by Spinks and Young (1958) originated from their observation that secondary and tertiary aliphatic amines in which the basic group is sterically hindered by attachment to a tertiary carbon atom have a modest level of ganglion-blocking activity.

By contrast, we first studied structure-activity relationships amongst certain aminobicyclo[2,2,1]-heptanes which were lower homologues of



R=H or alkyl

aminobicyclo-[2,2,1]heptanes. N atom is exocyclic

bicyclo[3,2,1]azaoctanes. N atom is endocyclic

mecamylamine. During this work we unexpectedly encountered a chemical route to bicyclo[3,2,1]azaoctanes, isomeric compounds in which the nitrogen atom was endocyclic, instead of exocyclic. Structure-activity relationships amongst these new compounds led us to discover the ganglion-blocking activity of the structurally simpler, unbridged compound pempidine. This work, which has already been very briefly outlined (Lee, Wragg, Corne, Edge, and Reading, 1958), is now reported in some detail.

METHODS

Chemical

The first four isomers of mecamylamine examined in the present work (Table I) were prepared as follows: *N*-methyl-*d*(−)-isobornylamine was prepared by the method of Wegler and Frank (1936). *N*-methyl-*d*(+)-bornylamine was prepared by the method of Forster (1899). *N*-methylfenchylamine was prepared in 20% yield by the Leuckhart reaction on fenchone. Hydrochloride sublimed >300°. (Found: C, 64.4; H, 10.8; N, 6.9. Required for $C_{11}H_{21}N$, HCl: C, 64.8; H, 10.8; N, 6.87.) *N*-methyl-camphidine was prepared by the method of Auwers (1868).

Most of the remaining compounds were prepared from nitrobicyclo[2,2,1]heptenes obtained via classical Diels-Alder reactions. Mild catalytic reduction of these intermediates gave the corresponding nitrobicyclo[2,2,1]heptanes. Catalytic reduction under more drastic conditions gave the expected aminobicyclo[2,2,1]heptanes, which could be *N*-alkylated by well-established methods to give products which undoubtedly had the structures shown as (V), (VII), and (IX) in Table II (Lee and Wragg, 1960a).

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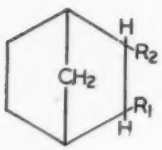
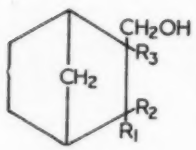
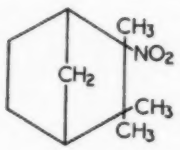


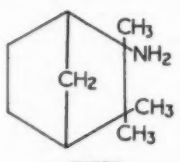
(X)



(XI)



(XII)



(XIII)

The compounds (X; $R_1 = -C_6H_5$, $R_2 = -NH.CH_3$, $-N(CH_3)_2$, or $-NH.CH_2.C_6H_5$) and (X; R_1 = cyclohexyl, $R_2 = -NH.CH_3$) were obtained similarly. The compounds (XI; $R_1 = R_2 = -CH_3$, $R_3 = -NH_2$) and (XI; $R_1 = -C_6H_5$, $R_2 = -H$, $R_3 = -NH.CH_3$) were synthesized via reactions between the appropriate nitrobicyclo[2,2,1]heptanes and formaldehyde. The compound (IV) in Table II was made by reducing the corresponding nitro-derivative chemically.

We were able to compare some of the foregoing aminobicyclo[2,2,1]heptanes (congeners of mecamylamine), in which the nitrogen atom is exocyclic, with the corresponding isomeric bicyclo[3,2,1]azaoctanes (structures (VI) and (VIII), in Table II; bridged congeners of pempidine), in which the nitrogen atom is endocyclic, as a result of a discovery made during a concurrent systematic study (Lee and Wragg, 1960b) of the aluminium lithium hydride reduction of crude 3-nitroisocamphane (XII). The basic fraction of the product mainly consisted of a single primary amine (M & B 4333) and, quite unexpectedly, two secondary amines (M & B 5561 and M & B 5562), all three of which were isomeric with the expected product (XIII).

The primary amine did not have the structure (XIII) because its *N*-methyl derivative (M & B 4334) was not identical with mecamylamine (I, Table II) (compare Stein, Sletzinger, Arnold, Reinhold, Gaines, and Pfister, 1956); moreover, both M & B 4333 and M & B 4334 were inactive when tested for ganglion-blocking activity and their actual chemical structures have not been investigated.

On the other hand, chemical evidence (Lee, Lunt, Wragg, and Barber, 1958; Lee, Wragg, Corne, Edge, and Reading, 1958) revealed that the two secondary amines had the structures (II; R=H) and (III; R=H) in Table II or geometrical isomers thereof. On the basis of chemical evidence, infra-red spectral data and the estimates of ganglion-blocking activity (see Results), we have provisionally assigned the structure (II; R=H) to M & B 5561 and the structure (III; R=H) to M & B 5562. Both compounds were *N*-methylated, giving M & B 5199 (II; $R = CH_3$) and M & B 5200 (III; $R = CH_3$) respectively, both of which are isomeric with mecamylamine. In practice, the two secondary amines formed in the aluminium lithium hydride reduction of (XII) were isolated as a mixture of 85% M & B 5561 and 15% M & B 5562, which could be separated into its components only by preparative gas chromatography. The unseparated mixture (M & B 4364) upon *N*-methylation gave M & B 4348A which consisted of 75% of the tertiary amine, M & B 5199, and 25% of its isomer, M & B 5200. M & B 4348 obtained in another experiment consisted of 55% M & B 5199 and 45% M & B 5200. These mixtures were studied as such because their separation into pure components by preparative gas chromatography was feasible only on a small scale.

We then proceeded to reduce with aluminium lithium hydride three nitrobicyclo[2,2,1]heptanes which had previously been reduced catalytically. Gas chromatography indicated that these reductions, in contrast to similar reduction of crude 3-nitroisocamphane (XII), each yielded only one product. The resulting single secondary amines were then *N*-methylated. Chemical and infra-red spectral evidence, together with the levels and duration of ganglion-blocking activity observed, led us to assign provisionally the symmetrical ring-enlarged structures (VI and VIII, analogous to II) (Table II) to the products thus obtained.

Pharmacological

The methods used were as described by Corne and Edge (1958), with the following exceptions and additions:

Most of the determinations of ganglion-blocking activity on the superior cervical ganglion-nictitating membrane preparation of the anaesthetized cat were performed using continuous (10/sec.) supramaximal pre-ganglionic stimulation. A few determinations were performed using intermittent (50/sec. for 5 sec./min.) stimulation. Several of the compounds had a short duration of action (comparable to that of hexamethonium). Greater reliance is placed on the potency estimates assigned to these compounds than to those with a long duration of action (comparable to that of mecamylamine), whose potency is difficult to estimate (Corne and Edge, 1958).

Acute oral and intravenous toxicities were studied in groups of 5 or 10 mice. Mydriatic responses in groups of 5 mice were observed after intraperitoneal, as well as oral, administration. Urinary excretion determinations were performed as described by Muggleton and Reading (1959).

The compounds were studied as the hydrohalides or acetates and all activity and toxicity determinations are expressed in terms of the cations. Other compounds used were hexamethonium bromide, acetylcholine chloride, adrenaline hydrochloride, histamine acid phosphate, nicotine hydrogen tartrate, and pilocarpine nitrate. With the exception of hexamethonium and adrenaline, all doses and concentrations of these compounds are expressed in terms of the salts.

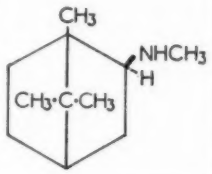
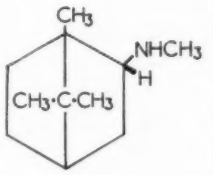
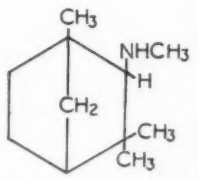
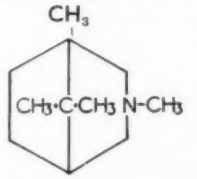
RESULTS

Four Structural Isomers of Mecamylamine

The activity of these compounds when tested on the pre-ganglionically stimulated nictitating membrane of the cat was only 4 to 7% of that of hexamethonium (Table I). None of these results was thought worth following up. A compound with an identical planar formula to *N*-methyl-*d*(−)-isobornylamine and *N*-methyl-*d*(+)-bornylamine but having an unspecified steric structure was reported by Rubinstein, Pedersen, Fakstorp, and Rønnov-Jessen (1958) to have a weak sympathetic ganglion-blocking activity and to be mildly hypertensive in the anaesthetized cat. A compound with an identical planar formula to *N*-methylfenchylamine but prepared by an unspecified route was also reported by Rubinstein *et al.* (1958) to have appreciable sympathetic ganglion-blocking activity. As our compound was short-acting and that studied by Rubinstein *et al.* was long-acting, it is unlikely that the two were identical chemically.

TABLE I

BLOCKING ACTIVITY OF 4 ISOMERS OF MECAMYLAMINE ON THE PRE-GANGLIONICALLY STIMULATED NICTITATING MEMBRANE OF THE CAT

Compound	Structure	Activity (Hexamethonium = 100)
N-Methyl-*d*-(−)-isobornylamine		5
N-Methyl-*d*(+)-bornylamine		4
N-Methylfenchylamine		7
N-Methylcamphidine		6

Aminobicyclo[2,2,1]heptane Derivatives (N Atom Exocyclic, as in Mecamylamine)

All of the phenyl and cyclohexyl derivatives of structures (X) and (XI) above had a very low activity, less than 3% of that of hexamethonium.

The ganglion-blocking activity, duration of action, and oral and intravenous toxicity of the nor derivatives of mecamylamine (structures IV, V, VII, and IX) are shown in Table II. Of these compounds, only two (M & B 4086 and M & B 4620) had a prolonged duration of action on the pre-ganglionically stimulated nictitating membrane, and M & B 4086 was selected for a brief examination of its mode of action.

Properties of M & B 4086.—Mecamylamine and M & B 4086 act specifically at the ganglion since a large intravenous dose (8 mg./kg.) of either compound completely inhibited the effect of pre-ganglionic stimulation on the nictitating membrane, but had no inhibitory action on post-ganglionic stimulation or on intravenously injected adrenaline (Fig. 1).

On the isolated guinea-pig ileum, M & B 4086 in a concentration of 0.08 mg./ml. completely inhibited the effect of nicotine-induced contractions and caused a slight reduction in the response to acetylcholine but had no effect on histamine- or pilocarpine-induced contractions. The nicotine response was slow to recover after removal of the antagonist from the bath. A concentration of 0.8 mg./ml. had an initial stimulant action and blocked the effect of all four agonists.

M & B 4086 had about 50% of the activity of, but a similar duration of action to, mecamylamine when compared by the mydriatic response in mice after intraperitoneal injection.

An oral dose of 5 mg./kg. in a group of six rats showed an identical urinary excretion pattern with that shown by mecamylamine over a period of 24 hr. Thus recoveries of M & B 4086 at 2, 4, 6, and 24 hr. were 3.3, 9.6, 16.5, and 48.7% respectively. The corresponding figures for mecamylamine were 1.3, 8.8, 15.2, and 45.6%.

TABLE II

BLOCKING ACTIVITY ON THE PRE-GANGLIONICALLY STIMULATED NICTITATING MEMBRANE OF THE CAT AND TOXICITY IN MICE OF SOME AMINOBICYCLO[2,2,1]HEPTANE AND BICYCLO[3,2,1]AZAOCTANE DERIVATIVES

VS = Very short duration of action (shorter than hexamethonium). S = Short duration of action (similar to hexamethonium). M = Medium duration of action (longer than hexamethonium, shorter than pempidine). L = Long duration of action (similar to pempidine). Where the results were very variable, the range of activity is given in parentheses.

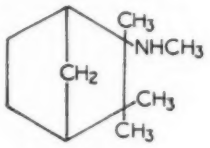
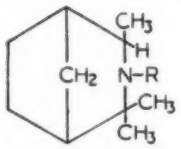
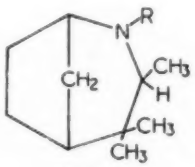
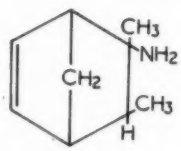
M & B No.	Structure	Activity (Hexamethonium = 100)	Number of Experiments	Duration of Action	LD50 mg./kg.	
					Oral	Intravenous
	Mecamylamine ←  (I)	120 (60–190)	5	L	98	12·9
5561	→ R = H	134 (85–170)	4	L		29·9
5199	R = CH_3 ←*isomers*  (II)	100 (50–200)	5	L		28·3
	isomers					
5562	→ R = H	75	3	VS		30·3
5200	R = CH_3 ←  (III)	40 (25–50)	5	M		32·5
4364	Mixture of 85% M & B 5561 and 15% M & B 5562	140 (120–190)	5	L	470	35·0
4348A	Mixture of 75% M & B 5199 and 25% M & B 5200	125	2	L	214	37·0
4348	Mixture of 55% M & B 5199 and 45% M & B 5200	170 (100–210)	6		L	
4315	 (IV)	15	1	S		

TABLE II—*continued*

M & B No.	Structure	Activity (Hexamethonium = 100)	Number of Experiments	Duration of Action	LD50 mg./kg. Oral	LD50 mg./kg. Intravenous
4058	$R_1=R_2=H$	15	2	S	≃135	46·0
4086	$R_1=CH_3$, $R_2=H$ ←	35	2	L	138	33·8
4620	$R_1=R_2=CH_3$	50	2	L	≃165	18·1
4126	$R_1=CH_2.C_6H_5$, $R_2=H$	<1	1			
	(V)					
4387	←*isomers*	50	3	L	≃415	48·5
	(VI)					
4188	→ $R=CH_3$	25	2	S		68·5
4269	$R=C_2H_5$←	25	2	S		34·2
	isomers *isomers*					
	(VII)					
4443	→ $R=CH_3$	25	2	S	≃425	109
4442	$R=C_2H_5$←	20–25	2	S	≃267	54·5
	(VIII)					
4280	←	15	2	S	405	44·5
	(IX)					

Bicyclo[3,2,1]azaoctane Derivatives (N Atom Endocyclic, as in Pempidine)

The activity, duration of action, and toxicity of these compounds (structures II, III, VI, and VIII) are shown in Table II. One of the two isomers in each of the mixtures designated M & B 4364 and M & B 4348A had a higher activity and a longer duration of action than the other, and this finding considered together with the results obtained amongst mecamylamine congeners strengthened the chemical and infra-red spectral evidence in assigning the structures as shown. The effects of

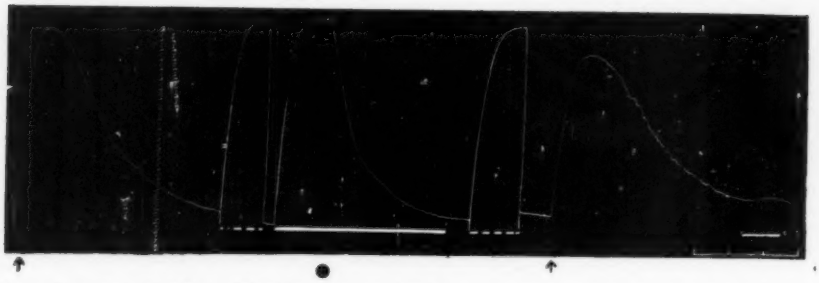


FIG. 1.—Cat, 2.4 kg., chloralose anaesthesia. Contractions of nictitating membrane. At arrows, 10 μg. adrenaline was injected intravenously; the continuous lines indicate pre-ganglionic stimulation at 10/sec.; the discontinuous lines indicate post-ganglionic stimulation at 10/sec.; at the black dot, 8 mg./kg. M & B 4086 was injected intravenously. Time, 60 sec.

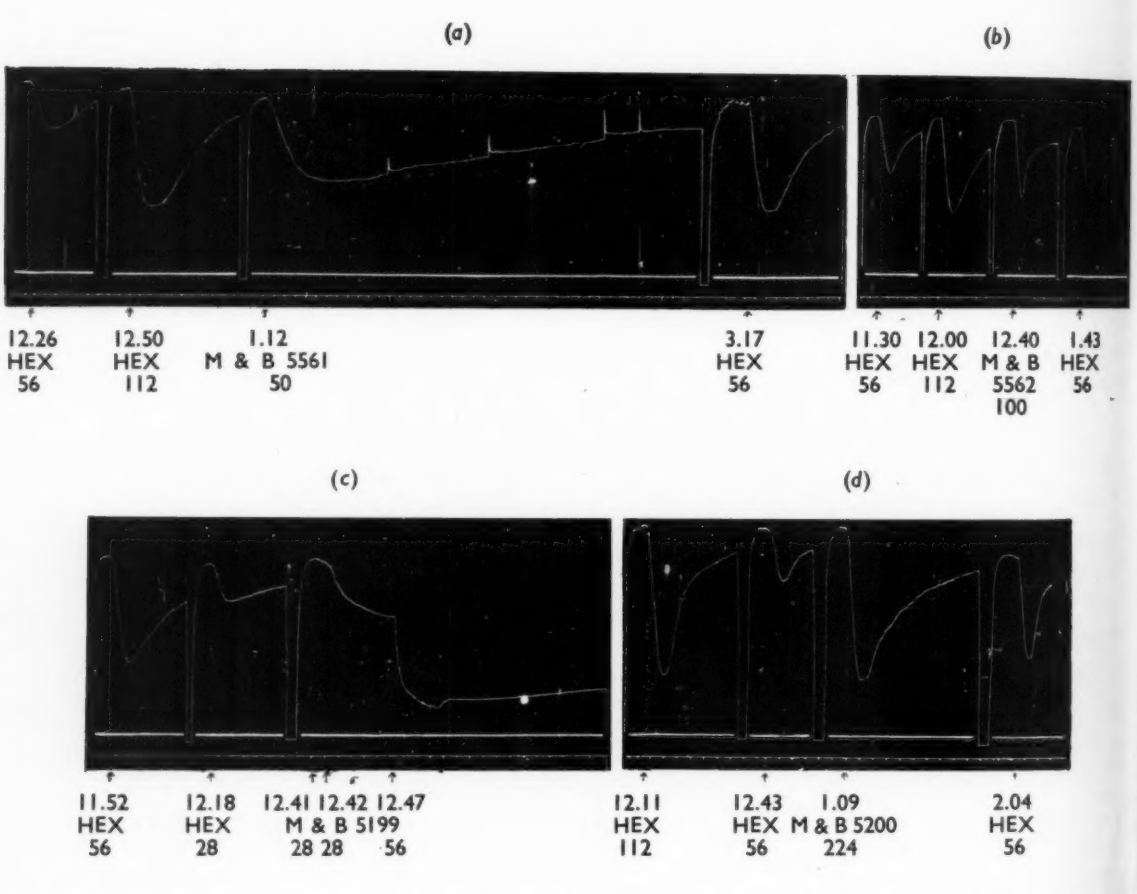


FIG. 2.—Cats, (*a*) 3.25 kg., (*b*) 2.5 kg., (*c*) 3.0 kg., (*d*) 2.3 kg., chloralose anaesthesia. Contractions of nictitating membrane. Continuous line indicates pre-ganglionic stimulation at 10/sec. Kymograph was stopped between periods of stimulation. Intravenous doses in μg./kg. at times shown. HEX = hexamethonium. Time, 60 sec.

these two pairs of isomers in comparison with hexamethonium on the pre-ganglionically stimulated nictitating membrane are shown in Fig. 2. When compared with hexamethonium on the pupillary diameter of the anaesthetized cat after removal of the superior cervical ganglion, M & B 5199 and M & B 5200 had activities of 250% and 70% respectively. When compared with mecamylamine in mice injected intraperitoneally, respective mydriatic activities of 100% and 50% were obtained. Some of the pharmacological properties of the mixture (M & B 4348A) of these two isomers were examined in more detail.

Properties of M & B 4348A.—An intravenous dose of 6.7 mg./kg. of M & B 4348A completely blocked the effect on the nictitating membrane of pre-ganglionic stimulation but had no effect on the contraction produced by post-ganglionic stimulation or by an intravenous injection of adrenaline. In the same preparation a further dose of 6.7 mg./kg. caused a small rise in blood pressure. In other experiments the pressor response to nicotine (0.1 mg./kg.) was reduced about 50% by a dose of 0.05 mg./kg., and 0.1 mg./kg. caused an almost total inhibition of the effect on the blood pressure of peripheral vagal stimulation. Doses of up to 6.7 mg./kg. had no effect on blood pressure responses to small doses of acetylcholine or histamine, and the response to adrenaline was potentiated. Respiratory arrest occurred after a total dose of 31 mg./kg. On the isolated Langendorff rabbit heart preparation doses of 2.0 to 8.0 mg. injected into the aortic cannula caused a slowing of the beat.

Oral doses of 3.3 mg./kg. and 4.0 mg./kg. of M & B 4348A and M & B 4364 respectively caused a marked mydriasis in mice within 10 min. of administration and the response was of prolonged duration.

On the isolated guinea-pig ileum a concentration of 0.067 mg./ml. of M & B 4348A completely inhibited the response to nicotine and reduced the response to pilocarpine, but had no effect on acetylcholine or histamine responses (Fig. 3). The compound was removed after being in contact with the gut for 14.5 min. and 50% recovery of the nicotine response occurred about 3 hr. later (Fig. 3).

On the isolated frog rectus abdominis muscle a concentration of 0.67 mg./ml. inhibited responses to acetylcholine without causing a contracture.

When examined for its potency on nicotine-induced convulsions in mice M & B 4348A injected intraperitoneally was found to have an ED50 of 0.065 mg./kg. The lower, less active, homologue (VI; M & B 4387) had an ED50 of 0.25 mg./kg. Consequently, these two compounds had respectively 1.66 and 0.43 times the activity of mecamylamine (ED50=0.108 mg./kg.) and 23 and 6 times the activity of hexamethonium (ED50=1.5 mg./kg.) in this test. The greater potency of amine, as compared with quaternary, ganglion-blocking compounds in this test has been described for pempidine by Corne and Edge (1958), and Stone, Meckelnburg, and Torchiana (1958) have found that peripheral ganglion-blocking activity and anticonvulsant potency are not necessarily related in structurally dissimilar compounds.

Discussion

Considering first the structure-activity relationships observed amongst mecamylamine congeners (IV, V, VII, and IX in Table II) it is apparent that the presence of a double bond in the 5,6-position had no effect on activity (compare M & B 4315 and M & B 4058). Activity was also unaffected by the substitution of a *C*-ethyl for a *C*-methyl group (compare M & B 4188 and M & B 4269). On the other hand, replacement of the *N*-methyl group in M & B 4086 by an *N*-benzyl group (M & B 4126) eliminated activity. Removal of one *C*-methyl group from the 3-position of mecamylamine (I) giving M & B 4280 (IX) resulted in a pronounced drop in activity and in a shortened duration of action. Rubinstein, Pedersen, Fakstorp, and Rønnov-Jessen (1958) have reported that a compound with this planar

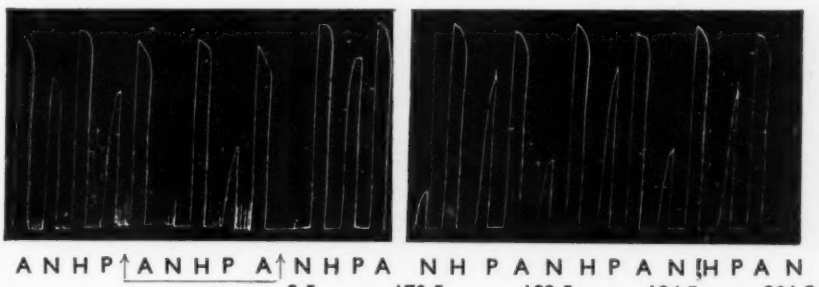


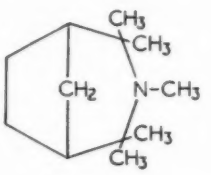
FIG. 3.—Contractions of isolated guinea-pig ileum in a 10 ml. bath. At A, 0.15 μg./ml. of acetylcholine; N, 5.0 μg./ml. of nicotine; H, 0.5 μg./ml. of histamine; P, 0.5 μg./ml. of pilocarpine. Between arrows 67 μg./ml. of M & B 4348A was present. Time in min. after removal of antagonist from the bath shown below nicotine responses.

formula, but prepared by an unspecified route, paralleled mecamylamine closely in sympathetic ganglion-blocking activity and in having a long duration of action. The pharmacological properties of our product make its chemical identity with the compound studied by Rubenstein *et al.* (1958) unlikely. By contrast, removal of a *C*-methyl group from the 2-position of (I) giving M & B 4086 (V ; $R_1 = -CH_3$, $R_2 = -H$) whilst lowering activity did not shorten the duration of action. The corresponding primary amine, M & B 4058, was less active than M & B 4086, whereas the corresponding tertiary amine, M & B 4620, was similar both in activity and in duration of action to M & B 4086. A similar level of activity in related secondary and tertiary amines was later encountered again, both in the endocyclic series (for example M & B 5561 and M & B 5199), and in pempidine and the corresponding secondary amine (Corne and Edge, 1958 ; Spinks *et al.*, 1958).

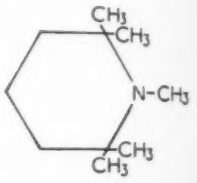
Of the two possible structures (II ; $R = -CH_3$) and (III ; $R = -CH_3$) for M & B 5199, the former appears more likely because an endocyclic nitrogen atom attached on each side to alkyl-substituted carbon atoms is a grouping which, in pempidine and its congeners, has since been found to be associated frequently with a high level of ganglion-blocking activity. Since infra-red spectral analysis eliminated the possibility that M & B 5200 is a geometrical isomer of M & B 5199, it probably has the structure (III ; $R = CH_3$). The fact that M & B 5200 is not only less active but also has a shorter duration of action than M & B 5199 is not incompatible with the suggested assignment of structure, because subsequent work has indicated that an adjacent bridge-end α-carbon atom is less likely to confer ganglion-blocking activity on an endocyclic nitrogen atom than is a gem-dimethyl group.

Perhaps the most significant implication of our results as a whole was that it had been possible to pass from the aminobicycloheptane structure, in which the nitrogen atom is exocyclic, to the isomeric bicycloazaoctane structure, in which the nitrogen atom is endocyclic, without loss in ganglion-blocking activity. Moreover, the biological results (Table II) on the pairs of isomers, M & B 4269 and M & B 4442, M & B 4188 and M & B 4443, M & B 4086 and M & B 4387, and mecamylamine and M & B 5199, conformed to a remarkably systematic pattern. In the first place, the individual compounds in each pair have both a closely similar level of ganglion-blocking activity and a similar duration of action, independent of whether the *C*-substituents are methyl or ethyl groups. Secondly, the successive introduction of *C*-methyl groups into M & B 4188 or M & B 4443 results in a stepwise increase both in activity and in duration of action in the resulting homologous series.

It thus became apparent that a high level of ganglion-blocking activity and a long duration of action would probably be exhibited by (XIV) in which the introduction of methyl groups surrounding the endocyclic nitrogen atom had been taken one step further than in M & B 5199.



(XIV)



(XV)

In practice we decided to synthesize instead the corresponding unbridged compound, pempidine (XV), which did in fact exhibit high activity and prolonged action.

We are indebted to Dr. H. J. Barber and Dr. R. Wien for their constructive interest in this work ; to Mr. A. C. Rasmussen for several estimations ; to Dr. H. W. Reading for carrying out the excretion experiments in rats ; to Dr. D. F. Muggleton for interpretation of infra-red spectra ; and to Dr. G. A. P. Tuey for separation and analysis of many samples by gas chromatography.

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CENTRAL VASOMOTOR EFFECTS OF A NEW GANGLION-BLOCKING AGENT—1 : 2 : 2 : 6 : 6-PENTAMETHYL PIPERIDINE (PEMPIDINE)

BY

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(RECEIVED JANUARY 12, 1960)

The central vasomotor effects of pempidine were studied in dogs and cats anaesthetized with pentobarbitone sodium, and in spinal cats. Pempidine lowered the threshold of electrical stimulation of the medulla oblongata to evoke a pressor response when given intravenously to cats in a dose insufficient to block autonomic ganglia. Injection of a small dose into the cerebral ventricles of dogs produced an increase in the pressor responses to occlusion of the carotid artery and to electrical stimulation of the central end of the cut vagus. In the spinal cat small doses of pempidine, given either intravenously or intrathecally, augmented the rise in blood pressure resulting from compression of the spinal cord. Since all these effects were produced by pempidine in doses insufficient to cause ganglionic block, the observed effects are attributed to central facilitation of the vasomotor responses. Thus pempidine has a stimulant action on the spinal and supraspinal centres.

It is known that piperidine derivatives block ganglionic transmission ; for example, Zamboni and Borghetti (1953) reported ganglion-blocking properties of N-ethyl piperidine. The pharmacology of 1:2:2:6:6-pentamethyl piperidine (pempidine) was independently reported by Spinks and Young (1958) and Corne and Edge (1958). This drug has a prolonged hypotensive effect attributed to strong and persistent ganglionic blockade. Harrington, Kincaid-Smith and Milne (1958) have shown that pempidine on parenteral administration passes through the blood-brain barrier and is then taken up by the brain. The present study was undertaken to determine possible central vasomotor effects of pempidine, independent of its ganglion-blocking action.

The main difficulty in the study of the central vasomotor effects of ganglion-blocking agents is that peripheral ganglionic blockade masks the central vasomotor effects of these agents (Dontas and Nickerson, 1956). To overcome this difficulty the drug was injected into the cerebral ventricles or intrathecally, or it was given intravenously in a dose too small to produce ganglionic blockade.

METHODS

The investigation was carried out in dogs and cats anaesthetized with pentobarbitone sodium (30 mg./kg. intravenously in dogs, and 35 mg./kg. intraperitoneally in cats). The animals were maintained on artificial respiration after cutting both vagi. Blood pressure was recorded from the left carotid artery. The intravenous injections were made through a polythene tube into a femoral vein, the intraventricular injections were made into the lateral ventricle as described by Bhargava and Tangri (1959), and the intrathecal injections through a needle inserted in the vertebral canal at the lumbosacral articulation.

The action of the drug on the brain stem was assessed by observing the effects on (*a*) the reflex pressor response obtained by occlusion of the right carotid artery, and by electrical stimulation of the central cut end of the right vagus nerve (by means of an electronic stimulator), and (*b*) on the pressor responses evoked by direct electrical stimulation of the medulla oblongata by means of a bipolar needle electrode using a stereotaxic technique. The electrode placement was aided by the parameters described by Wang and Ranson (1939). The site of action of the drug in the spinal cord was assessed by observing the effects on the vasomotor responses elicited by the technique of spinal compression in the spinal cat (Bhargava and Kulsreshtha, 1959). Contractions of the nictitating membrane were obtained by preganglionic stimulation of the cervical sympathetic nerve, and pressor ("nicotinic") responses by injection of acetylcholine after atropine.

Pempidine was used as the hydrochloride.

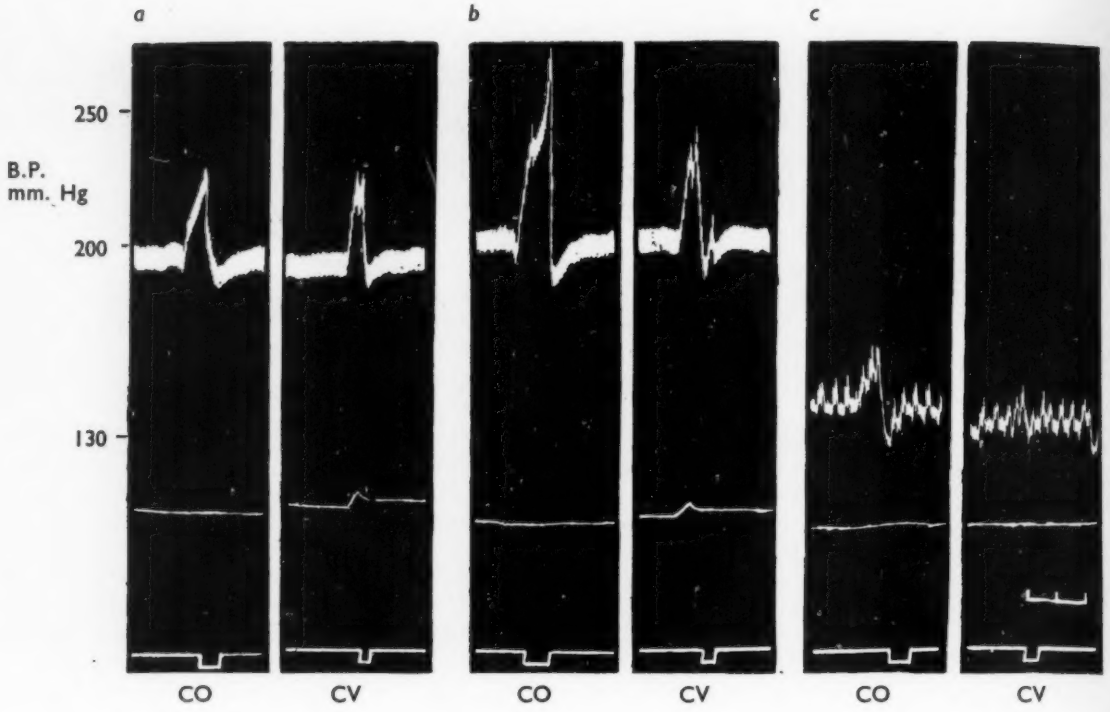

FIG. 1.—Records of arterial blood pressure (upper tracing) and of the nictitating membrane (lower tracing) of a dog, 7.2 kg., anaesthetized with pentobarbitone sodium. CO, response to 30 sec. occlusion of the carotid artery. CV, response to electrical stimulation (4 V, 10 msec., 10/sec. for 10 sec.) of cut end of the central stump of the vago-sympathetic nerve. (*a*) Control, (*b*) 10 min. after intraventricular injection of 50 μg. of pempidine, (*c*) 15 min. after intravenous injection of 0.5 mg./kg. of pempidine. Time signal in 30 sec.

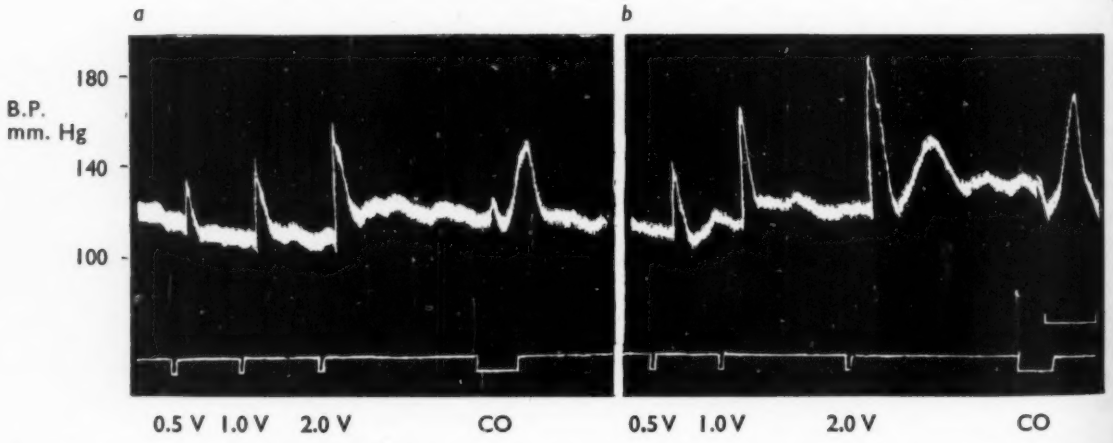

FIG. 2.—Record of arterial blood pressure of a cat, 3.0 kg., anaesthetized with pentobarbitone sodium. CO, response to 30 sec. occlusion of the carotid artery. 0.5 V, 1.0 V, 2.0 V; electrical stimulation of the medulla oblongata by means of a bipolar electrode using a stereotaxic technique. (*a*) Control, (*b*) 15 min. after intravenous injection of 25 μg. pempidine. Time signal in min. Note potentiation of all the vasomotor responses.

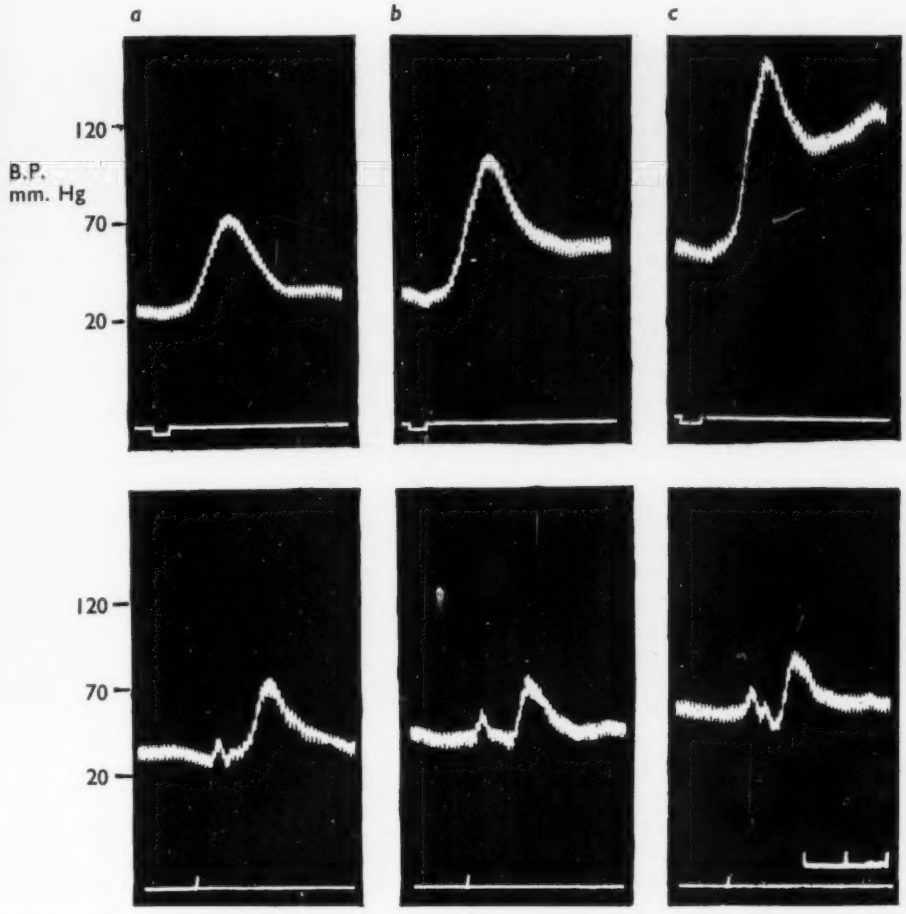

FIG. 3.—Record of arterial blood pressure of a spinal cat, 3.0 kg. Upper tracing shows vasomotor responses to compression of the spinal cord (150 mm. Hg for 10 sec.). Lower tracing shows " nicotinic " pressor responses to 150 μg. acetylcholine after atropine. Time signal in min. (*a*) Control, (*b*) 20 min. after intravenous injection of 25 μg./kg. of pempidine, (*c*) 10 min. after intrathecal injection of 5 μg./kg. of pempidine. Note the increased responses to spinal compression whereas those to acetylcholine were unaltered.

RESULTS

The Reflex Vasomotor Responses in the Dog After Injection of Pempidine into the Cerebral Ventricles

The effects of intraventricular administration of pempidine (50 to 100 μg.) on reflex supraspinal vasomotor responses were studied in 3 dogs. The results of one experiment are shown in Fig. 1. The control records (CO) show the pressor responses to occlusion of the carotid artery for 30 sec. (CO), and the pressor response as well as the nictitating membrane contraction obtained by electrical stimulation of the central cut end of the vago-sympathetic trunk (CV). On intraventricular administration of 50 μg. pempidine the blood pressure rose about 10 mm. Hg, and both the reflex vasomotor responses increased. The pressor response to occlusion of the carotid artery increased from 30 to 80 mm. Hg, and the response to central stimulation of the vagus increased from 30 to 45 mm. Hg. The contraction of the nictitating membrane was unchanged. The subsequent intravenous injection of 0.5 mg./kg. pempidine produced a marked fall of arterial blood pressure of 50 mm. Hg, and abolished both the reflex vasomotor responses (CO and CV). The nictitating membrane response was also abolished, therefore the inhibition of the vasomotor responses can be attributed solely to ganglionic blockade.

The Vasomotor Responses in the Cat After Intravenous Injection of Pempidine

The results were essentially the same in 3 cats. Fig. 2 illustrates the graded pressor responses evoked by electrical stimulation of the medulla with increasing voltage and the pressor response to the 30 sec. occlusion of the carotid artery (CO). Fifteen min. after the intravenous injection of pempidine (25 μg./kg.) all these vasomotor responses were increased. The control response to 1.0 V was now obtained with 0.5 V, and that to 2.0 V with 1.0 V. Thus the threshold of medullary excitability was lowered after the pempidine administration.

Spinal Vasomotor Responses After Pempidine in the Spinal Cat

The effect of pempidine on the spinal vasomotor responses was studied in 8 cats after intravenous injection of 25 to 50 μg./kg. and in 3 of these also after intrathecal administration of 5 μg./kg. Fig. 3 shows the vasomotor responses to spinal compression (upper record), and the "nicotinic" pressor responses to 150 μg. of intravenous acetylcholine after atropine (lower record). After intravenous administration of pempidine (25 μg./kg.) the blood pressure rose 10 mm. Hg. Twenty min. later, the vasomotor response to spinal cord compression was increased. When 5 μg./kg. of pempidine was given intrathecally after 1 hr. and the spinal cord was then compressed 10 min. later, the vasomotor response was further increased. This augmentation of the response persisted for 1.5 hr.

After intravenous injection of 25 μg./kg. pempidine the augmented response to compression of the spinal cord appeared within 10 to 20 min. and persisted for 1.5 hr. In one cat in which 50 μg./kg. was injected intravenously the blood pressure was lowered and the response to compression of the spinal cord was depressed within 10 min. and abolished within 30 min., but this also occurred with the response to acetylcholine. In this experiment this dose of pempidine must, therefore, already have exerted its effect on ganglionic transmission.

Discussion

The results of the present investigation showed that pempidine has a central vasomotor stimulant effect at the supraspinal as well as at the spinal level. This central effect which was examined on reflex vasomotor responses is best seen when the drug is injected into the cerebral ventricles or intrathecally. On intravenous administration it can only be seen when the dose injected is too small to produce ganglion-block.

A pressor action of pempidine resulting from large intravenous doses has been reported by Corne and Edge (1958) in cats with a low blood pressure. The pressor action was not blocked by bilateral adrenalectomy or by administration of phenoxybenzamine. However, the observation that the pressor response was considerably reduced after spinal cord destruction suggests that the effect was at least partly a result of a central action. The observation that pempidine in doses insufficient to block the ganglia manifests a central vasomotor stimulation may have a bearing on its clinical use. Small doses of pempidine may raise the blood pressure by its central action, but with adequate dosage hypotension must be produced by its ganglion-blocking effect.

We would like to thank Dr. R. Wien of May and Baker Ltd., Dagenham, Essex, for the supply of pempidine.

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THE INHIBITION OF THE PERISTALTIC REFLEX BY SUBSTANCES FROM PROTEIN SOURCES

BY

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An aqueous extract of gluten, and its ultrafiltrate, depressed the peristaltic reflex of the rat jejunum. This effect was shared by similar extracts of mammalian skeletal muscle but not by such proteins as casein, gelatin, and albumin. Peptic-tryptic digestion did not abolish the effect, whereas acid hydrolysis of the active fractions destroyed the depressant substance.

Radiographic abnormalities of the small intestine have been recognized for some years as one of the features of adult patients with idiopathic steatorrhoea (Snell and Camp, 1934; Kantor, 1939), and children with coeliac disease (Golden, 1936; Flemming Møller, Nørgaard and Plum, 1948). The classical appearance of clumped masses of opaque medium invariably seen in these patients was thought to be due to alternate areas of hypotonia and hypertonia resulting from disordered motor function attributed to vitamin B deficiencies or other factors (Golden, 1945). A number of workers had observed slow transit time in these patients, but this might have been due to increased tone, as well as to decreased motility. This rather confused situation was clarified by the demonstration that the clumped appearance of the barium sulphate was not due to alternate areas of spasm and relaxation, but to flocculation of the opaque medium due to the presence of excessive quantities of mucus (Frazer, French, and Thompson, 1949). Avoidance of flocculation by the use of non-flocculating media revealed that the characteristic change in these patients was dilatation (Anderson, Astley, French, and Gerrard, 1952).

Intolerance of dietary wheat gluten has since been established as the basic cause of the enteropathy in both adults and children. Wheat gluten caused deterioration in patients with gluten-induced enteropathy if it was reintroduced into the diet of patients in remission on a gluten-free régime (Dicke, 1950; Weijers, van de Kamer, and Dicke, 1953; Anderson, Frazer, French, Hawkins, Ross, and Sammons, 1954; Ross, Frazer, French, Gerrard, Sammons, and Smellie, 1955; Frazer, Fletcher, Ross, Shaw, Sammons, and Schneider, 1959). A change from the normal feathery pattern to dilatation was observed (Anderson, Frazer, French, Gerrard, Sammons, and Smellie, 1952).

It was, therefore, of interest to study the effect of gluten, or fractions derived from it, on isolated intestinal preparations. This paper is concerned with the examination of the effect of gluten and gluten fractions, and similar preparations of other proteins, on the peristaltic reflex of rat small intestine.

METHODS

Peristaltic Reflex

All fractions were investigated for their effect on the peristaltic reflex of the isolated rat jejunum. Bülbring, Crema, and Saxby's modification (1958) of the Trendelenburg method was used. Fractions were applied to the serosal surface only.

Fractionation Procedures (Fig. 1)

Cold Aqueous Extraction.—Gluten (Energen Ltd.) from untreated wheat flour is only poorly soluble in water. An aqueous extract (A.E.) was prepared by shaking 20 g. of gluten with 200 ml. of water for 30 min. at room temperature. The suspension was centrifuged at 3,000 rev./min. for 20 min., the opalescent supernatant freeze-dried and the resultant solid used. An ultrafiltrate (A.E./U.F.) was made from the supernatant using Visking dialysis tubing (Grant, Rowe, and Stanworth, 1958).

Hot Aqueous Extraction.—This method consisted in homogenizing 20 g. of gluten in water and boiling for 20 min. to coagulate most of the protein. The mixture was then cooled and subjected to ultrafiltration. The ultrafiltrate was freeze-dried as above (A.E.H./U.F.).

Peptic-tryptic Digestion.—A peptic-tryptic digest (III) was prepared as described by Frazer, Fletcher, Ross, Shaw, Sammons, and Schneider (1959). This was further treated either by autoclaving (Frazer,

Fletcher, Ross, Shaw, Sammons, and Schneider, 1959) which denatures protein, or by ultrafiltration which removes proteins of molecular weight over 20,000. Filtrates from these processes (III A.F. and III U.F. respectively) were used in the biological investigations. The separate peptic (I) and tryptic (II) digests were not prepared.

Acid Hydrolysis.—Fractions A.E., III U.F. and III A.F. were hydrolysed by boiling 1.0 g. material with 500 ml. 6N hydrochloric acid overnight. The acid was removed by distillation *in vacuo* at 50 to 60°; water was added to the residue and the distillation repeated. Addition of water and subsequent distillation were then repeated twice. The final residue was taken up in water and the *p*H adjusted to 7.

Mammalian Skeletal Muscle.—Lean beef trimmed of excess fat was used as the source of mammalian

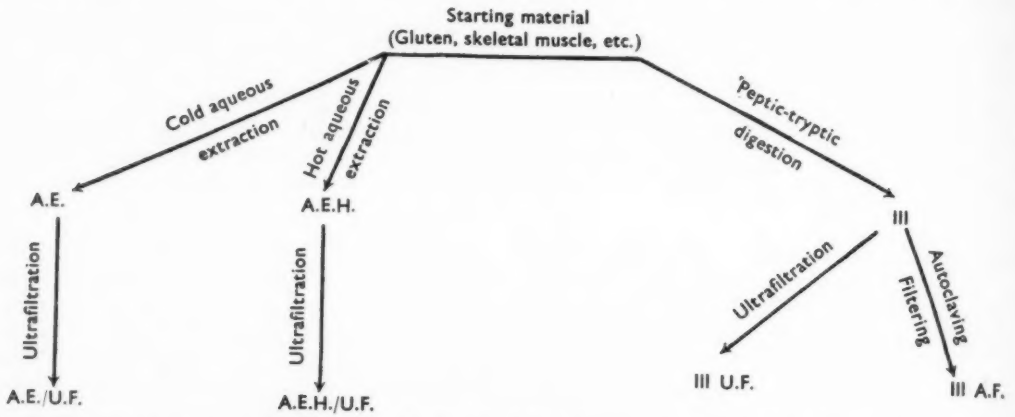

FIG. 1.—Diagram to illustrate the methods used to prepare the various fractions.

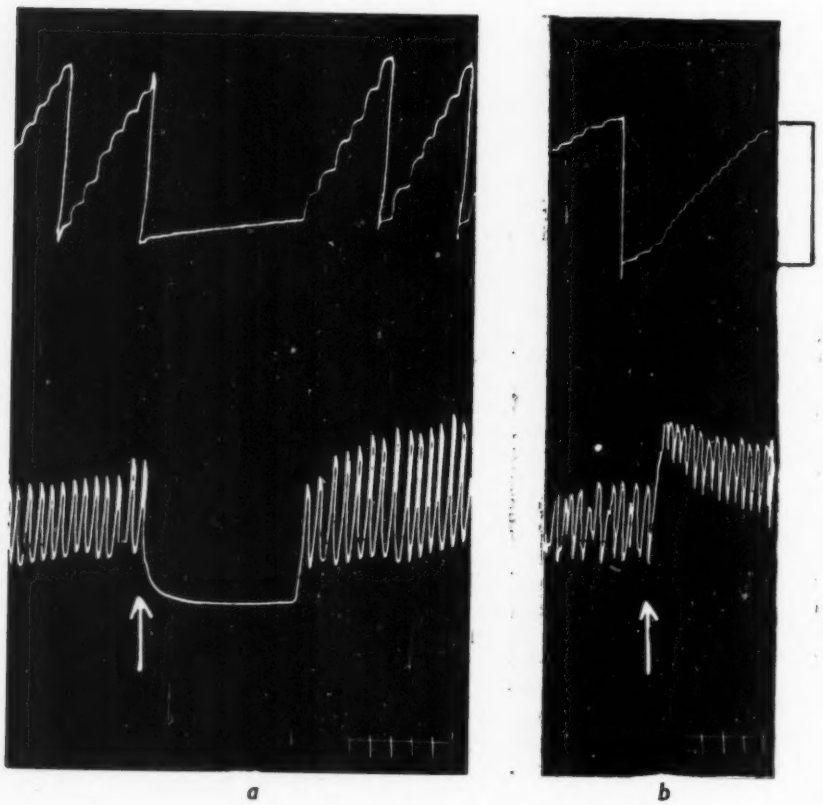

FIG. 2.—Isolated rat jejunum. Upper tracing: volume of fluid expelled during peristaltic reflex. Lower tracing: contractions of the longitudinal muscle. At (a) 2.0 mg./ml. of an autoclaved peptic-tryptic digest of gluten. At (b) 2.0 mg./ml. of an acid hydrolysate of an aqueous extract of skeletal muscle. Time, 30 sec. Doses were applied to the serosal surface.

skeletal muscle. Fractions A.E., A.E./U.F., A.E.H./U.F., III and III A.F. were prepared as described above for gluten.

Chemicals

Pure Proteins.—Casein and gelatin (British Drug Houses) and albumin (bovine plasma, fraction V, Armour laboratories) are all water soluble; samples from 10% aqueous solutions were used without further purification. In addition the following fractions were tested: casein A.E./U.F. and casein III and III A.F., and gelatin III and III A.F.

Peptides.—Glutathione, glycylglycine (British Drug Houses) and glycylglycylglycine (Nutritional Biochemicals Corporation, Cleveland, Ohio) were tested without further purification. Paper chromatography of these peptides showed no evidence of any ninhydrin-reacting impurities.

Amino Acids.—Glycine, (−)-proline, (−)-glutamic acid, (−)-tryptophan (L. Light and Co. Ltd.), (+)-glutamic acid and (−)-asparagine (British Drug Houses) were also tested.

RESULTS

Effect of Peptic-tryptic Digests of Gluten.—As the primary interest of this investigation was the possible inhibitory effect of digests of gluten on the peristaltic reflex, the following procedure was adopted. The isolated intestine was first tested for its response to 2.0 mg./ml. of the autoclaved filtered peptic-tryptic digest of gluten (III A.F.).

TABLE I

EFFECT OF FRACTIONS OF GLUTEN AND OTHER PROTEIN SOURCES ON THE PERISTALTIC REFLEX OF THE RAT JEJUNUM

Doses in mg./ml. Number of experiments shown in parentheses.

Material	Aqueous Extract		Ultrafiltrate of Aqueous Extract		Peptic-tryptic Digest	Ultrafiltrate of Peptic-tryptic Digest		Filtrate of Autoclaved Peptic-tryptic Digest	
	A.E.		A.E./U.F.	A.E.H./U.F.	III	III U.F.		III A.F.	
	Untreated	Acid Hydrolysate	Untreated		Untreated	Untreated	Acid Hydrolysate	Untreated	Acid Hydrolysate
Gluten	Inhibition 0·2–2·0 (3)	No effect 2·0 (1)	Inhibition 0·02–0·4 (33) No effect 0·02 (1)	Inhibition 0·2–0·4 (5)	Inhibition 2·0 (1)	Inhibition 0·2–2·0 (5) Doubtful 0·4 (1)	No effect 2·0 (2) Doubtful 3·2 (1)	Inhibition 1·5–2·0 (7)	No effect 2·0 (3)
Skeletal muscle	No effect 0·4–1·0 (2) Inhibition 2·0 (4) Doubtful 2·0 (1)	Stimulation 2·0 (1)	No effect 0·2–1·0 (5) Inhibition 2·0 (1)	Inhibition 0·2–1·0 (7)	Inhibition 2·0 (2) Doubtful 0·4 (1)			Inhibition 2·0 (2)	
Casein	No effect 2·0 (1)	Stimulation 2·0 (1)	No effect 2·0 (1)		Inhibition 2·0 (2)			Inhibition 2·0 (1) No effect 2·0 (1)	
Gelatin	No effect 2·0 (1)	Stimulation 2·0 (1)			No effect 1·9–2·0 (4)			No effect 2·0 (2)	
Albumin	No effect 2·0 (1)	Stimulation 2·0 (1)							

Inhibition usually occurred (Fig. 2a). Occasionally there was no response ; the intestine was then discarded. Frequently a slight increase of sensitivity to this inhibitory effect was observed during the course of the experiment. Inhibition occurred immediately on injecting the material into the bath. The preparatory and the emptying phase of the reflex were affected simultaneously, movements ceased and usually the baseline of the longitudinal muscle trace dropped. The duration of the effect varied with the sensitivity of the jejunum. With less sensitive preparations movements returned while the material was still in the bath ; with more sensitive preparations the bath fluid was changed, after the effect had lasted for 2 to 3 min. After washing, the preparation was rested for approximately 10 min., when movements invariably returned.

Effect of Aqueous Extracts of Gluten.—In order to obtain further information regarding the nature of the active material in gluten an aqueous extract of the unmodified protein was tested as well as various further modifications. The results are summarized in Table I. The aqueous extract of the whole protein (A.E.) was about 10 times more active than both the original peptic-tryptic digest (III A.F.), and the peptic-tryptic digest without treatment by autoclaving (III). Ultrafiltration of the aqueous extract and of the peptic-tryptic digest did not alter their activity.

Effect of Acid Hydrolysis on the Activity.—Acid hydrolysis completely abolished the activity of the whole protein, as well as of the peptic-tryptic digest after autoclaving and filtration, and of its ultrafiltrate.

Effect of Other Proteins on the Peristaltic Reflex.—A comparison with other proteins or protein-containing material was made in order to determine whether the inhibitory effect of gluten on the peristaltic reflex is specific for this substance. The results are shown in Table I. An aqueous extract of muscle (A.E.) as well as its ultrafiltrate (A.E./U.F.) depressed the peristaltic reflex slightly, but the activity was less than one-fifth of the corresponding gluten fraction. However, the ultrafiltrate of muscle extracts obtained by boiling (A.E.H./U.F.), as well as the peptic-tryptic digest, were as potent as the corresponding gluten fractions. After acid hydrolysis extracts of skeletal muscle did not inhibit but stimulated the intestine. This stimulation consisted of an increase in the rate of peristalsis and a rise of the baseline of the longitudinal muscle trace (Fig. 2b).

Solutions of the three pure proteins, casein, gelatin and albumin, showed no depressant activity in concentrations of 2 mg./ml. After peptic-tryptic digestion casein but not gelatin inhibited the peristaltic reflex. After acid hydrolysis the three pure proteins, like the skeletal muscle extracts, produced a stimulating effect.

Effect of Peptides and Amino-acids.—Six amino-acids, glycine, (−)-proline, glutamic acid ((+)- and (−)-), (−)-asparagine and (−)-tryptophan, as well as three peptides, glutathione, glycylglycine and glycylglycylglycine, were tested in concentrations of 1.0 to 2.0 mg./ml. In no experiment was there any depression of peristalsis. Occasionally a slight stimulating effect was observed. Tryptophan, the only amino acid that is destroyed by acid hydrolysis, showed such a stimulating effect.

Discussion

The results of this investigation show that some protein sources such as gluten and mammalian skeletal muscle contain water-soluble material that will inhibit the peristaltic reflex in the isolated rat intestine, when applied to the serosal surface. No inhibition was observed with casein, gelatin and albumin under the same conditions. The nature of the active material is as yet undetermined. It is not a large protein, as ultrafiltration, which removes molecules of a weight of over 20,000, did not reduce this effect. Digestion with pepsin and trypsin did not affect activity. This is further evidence that the material is not likely to be a whole protein. Moreover, in one experiment, peptic-tryptic digestion of casein led to the appearance of activity in previously inert material. This was possibly due to the preparation of smaller molecules. Furthermore, denaturation of the protein of such digests by autoclaving did not reduce their activity. The active principle is not a free amino acid, as acid hydrolysis completely abolished the depressant effect on the peristaltic reflex, and in some instances even led to the appearance of a stimulating action which was also exhibited by most of the pure amino acids. Attempts at further purification and identification are being carried out.

These studies are part of a collaborative programme of work on the pathogenesis of gluten-induced enteropathy which is being undertaken in this department under the general direction of Professor A. C. Frazer. We wish to thank Mrs. B. Matthews for technical assistance and the Energen Foods Co. Ltd. for supplies of gluten.

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A GANGLION-STIMULATING PRINCIPLE PRESENT IN PEPTONE

BY

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(RECEIVED FEBRUARY 16, 1960)

One of us (W. T. B.) has previously described a pressor effect of peptone in the rat. This effect was considered secondary to a release of sympathin by the stimulation of sympathetic ganglia. The present experiments show that peptone causes contraction of guinea-pig ileum and inferior eyelid of the rat which is antagonized by hexamethonium. Atropine inhibits equiactive doses of peptone and acetylcholine on the guinea-pig ileum and eserine potentiates them. Boiling peptone in concentrated hydrochloric acid or N sodium hydroxide destroys its activity; incubation with proteolytic enzymes (trypsin, pepsin, and chymotrypsin) has no effect on its activity. It is concluded that peptone acts upon the intramural ganglia of the guinea-pig ileum and on sympathetic ganglia which supply the inferior eyelid of the rat.

In a previous paper one of us described the pressor effect of peptone in the rat (Beraldo, 1958). The peptone-induced pressor effect was not abolished by adrenalectomy, but it was abolished by anti-adrenaline drugs and by paralysing doses of nicotine and hexamethonium. These experiments suggested that peptone stimulated sympathetic ganglia with the consequent release of sympathin.

In pursuing this investigation we have studied the effect of peptone on the guinea-pig ileum, the inferior eyelid of the rat and on the superior cervical ganglion of the cat. The results of these experiments are presented in this paper.

METHODS

Guinea-pig Ileum.—The preparation was suspended in 10 ml. aerated Tyrode solution at 35°. The contractions were recorded by means of a frontal writing lever. The substances employed to make the intestine contract were always used in submaximal dose. Doses were applied for 1 min., and then washed out; they were given at intervals of 2 to 3 min. Hexamethonium and cocaine were allowed to act for periods of up to 4 min.

Inferior Eyelid of the Rat.—This is a modification of the method described by Gertner (1956) for studying the effects of drugs acting on smooth muscle and sympathetic ganglia. Wistar rats weighing 350 to 450 g. were used. The animals were anaesthetized with ether followed by intravenous chloralose (12 mg./100 g.). A cannula was inserted into the trachea. The femoral vein was cannulated with a hypodermic needle mounted in a polythene tube. Heparin (50 to 100 units/100 g. body weight) was injected through the venous cannula. The lingual artery was similarly cannulated. The solutions, if not otherwise stated, were injected through the lingual artery by means of a 1 ml. tuberculin syringe and were washed in with 0.1 ml. of normal saline. The eyelid movements were recorded by means of a thread tied through the lower eyelid and fixed to an isotonic lever with a frontal writing point; the magnification was approximately 17 fold.

Nictitating Membrane of the Cat.—Cats were anaesthetized with ether followed by intravenous chloralose (80 mg./kg.). The lingual artery was cannulated with a hypodermic needle mounted in a polythene tube. All solutions were injected through the lingual artery. The nictitating membrane was connected to an isotonic lever which gave a magnification of about 10 fold.

Drugs Used.—Chloralose (Merck) 120 mg./10 ml. solution in saline. Heparin (Vitrum) 5% solution (5,000 units/ml.). Witte's peptone (Friedr. Witte, Rostock) 10 to 20% fresh solution in distilled water. The peptone solution was acidified with concentrated hydrochloric acid to *p*H 2 to 3, shaken with permutit (1 g. of permutit/g. of peptone), filtered and neutralized before use. This treatment removed histamine from the solution. Most of the experiments were performed with Witte's peptone, but other peptone preparations were also used (Pfanstiehl Peptone and Bacteriologic Peptone, Parke, Davis).

The quantities of hexamethonium bromide (Bistrium, Squibb), atropine sulphate (Poulenc Freres),

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1,1-dimethyl-4-phenylpiperazinium iodide (DMPP) (Parke, Davis), cocaine hydrochloride (Enila), acetylcholine hydrochloride (Roche) and eserine sulphate (Evans Medical Supplies Ltd.) used are expressed as weights of the salts. Nicotine (Schering-Kahlbaum) is given as the weight of the base. Chymotrypsin, crystallized salt free; and trypsin, 50% magnesium sulphate (Worthington Biochemical Sales Co.). Pepsin, 2 x crystallized (Nutritional Biochemical Corporation). Darco (activated carbon, Atlas Powder Co.) and permutit, Folin (Anachemia).

Results

Experiments on the Guinea-pig Intestine

When peptone in a dose of 20 to 40 mg. (0.1 to 0.2 ml. of a 20% solution) was added to the guinea-pig ileum, the muscle responded by a contraction in about 40% of the preparations. In these same preparations 0.01 to 0.02 μg. of acetylcholine usually produced a contraction of 3 to 5 cm. In the remaining 60%, peptone commonly failed to produce a response and the sensitivity to acetylcholine was correspondingly low; the effective dose of acetylcholine lay between 0.1 and 0.5 μg. When 0.5 to 1 μg. of eserine was added to the bath the sensitivity of the preparation to acetylcholine and to peptone increased by about 5 to 15 times.

Fig. 1 illustrates an experiment in which pronounced potentiation of the peptone response had occurred.

When atropine was added in a concentration which inhibited equiactive doses of peptone and acetylcholine, the recovery of the response to subsequent doses of the drugs followed a parallel course. This fact suggested that acetylcholine or some other ester of choline was a constituent of the peptone solution. Fig. 1 (B) shows the results obtained in one such experiment.

The possible presence of a choline ester in the peptone was investigated. 5 ml. of 10% peptone solution was adjusted with N sodium hydroxide to *p*H 8.5. Control experiments were performed with 5 ml. of acetylcholine solution (10 μg./ml.) at the same *p*H. Both solutions were left at room temperature (20°). After 1 hr. the *p*H of the solutions was readjusted to 7.4 and the activity of each solution was tested on the guinea-pig ileum. The activity of the acetylcholine solution had disappeared completely, but that of the peptone remained unaltered.

Eserinized guinea-pig ileum did not become desensitized or show tachyphylaxis on repeated additions of peptone to the bath over a period of 7 to 8 hr.

Mode of Action of Peptone.—The question remained whether or not peptone acted directly on the smooth muscle or on the nervous structures in the intestine.

Cocaine.—If peptone acted by stimulating nervous structures in the intestine, cocaine would be expected to abolish its effect. Feldberg (1951) has shown that cocaine in weak concentrations paralyses the nervous tissue of the gut, thereby abolishing the contractions to nicotine but not to histamine, the effect of which is actually increased. The fact that cocaine paralyses the nervous structures without reducing the response to histamine may be taken as evidence against a nervous component in the action of histamine on the intestine.

When in the present experiments the nervous structures were paralysed by cocaine, the ileum did not respond to nicotine and peptone, but

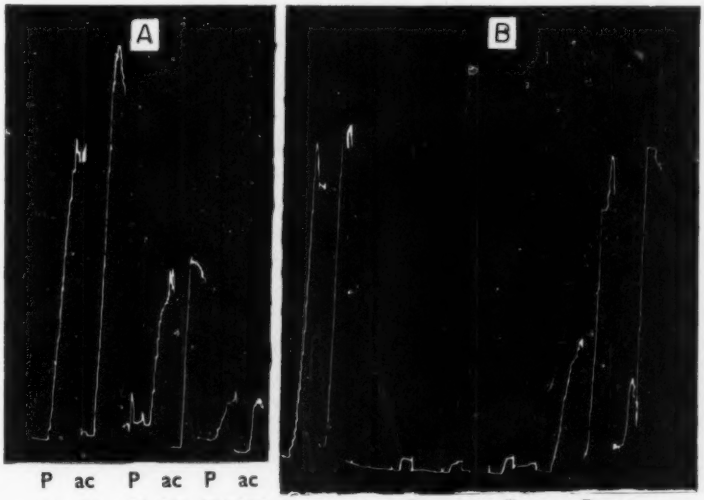

FIG. 1.—Guinea-pig ileum preparation in 10 ml. bath. A, Effect of 1 μg. eserine, added previously to the bath, on contractions caused by 40 mg. peptone (at P) and by 0.02 μg. acetylcholine (at ac). Time in min. shows the gradual reduction both of the acetylcholine and peptone response as the eserine potentiation disappears. B, Effect of 1 μg. atropine (1), allowed to remain in the bath for 1 min., on contractions caused by 40 mg. peptone (at P) and 0.02 μg. acetylcholine (at ac). 1 μg. eserine was maintained in the bath throughout the experiment. A period of 3 min. elapsed between (A) and (B). Acetylcholine and peptone were added to the bath at 2 min. intervals.

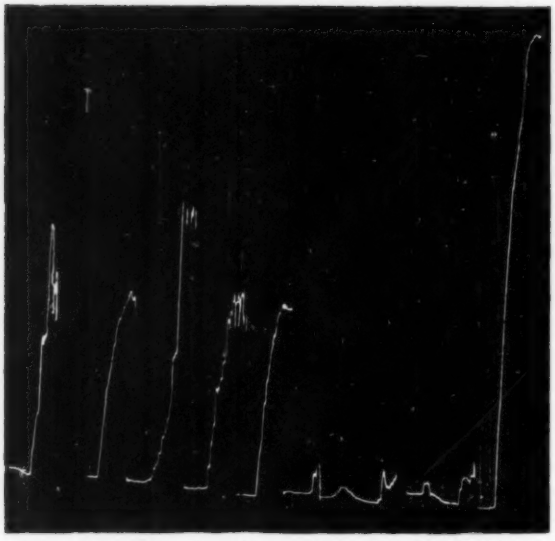

FIG. 2.—Guinea-pig ileum preparation in 10 ml. bath. Effect of 300 μg. cocaine (C) (allowed to remain in the bath for 2 min.) on the response to 20 mg. peptone (at P), 3 μg. nicotine (at Ni) and 0.1 μg. histamine (at H). The substances were added to the bath at 2 min. intervals.

histamine retained its full effect and at times produced a stronger contraction. The results of these experiments suggest that peptone acts on the guinea-pig intestine by stimulating nervous structures in the intestinal wall. This is illustrated in Fig. 2.

Hexamethonium.—Since equally strong contractions to peptone and nicotine were abolished by cocaine, the further question arose whether peptone acted by stimulating nerve cells or nerve fibres in the intestinal wall. An answer was sought by paralysing the ganglion cells of the myenteric plexus with ganglion-blocking agents such as hexamethonium. The effect of hexamethonium (100 μg. to 10 mg.) on the response to doses of peptone (10 to 20 mg.) was tested 22 times on 11 different preparations. The response was reduced in 17 of the tests, unchanged in 3 and augmented in 2. In 7 preparations the reduction was between 80 and 100%, and in 10 between 26 and 64%. The percentage reduction in the response to peptone was obtained by measuring the height of the contractions immediately before and during the action of hexamethonium. In order to test the effectiveness of ganglionic blockade after paralysing doses of hexamethonium, either 1,1-dimethyl-4-phenylpiperazinium iodide, a potent ganglion-stimulating drug studied by Chen, Portman, and Wickel (1951), or nicotine was used. When the responses of the intestine to equiactive doses of nicotine and peptone were blocked with hexamethonium, the recovery of sensitivity to subsequent doses of these drugs followed a parallel course, as shown in Fig. 3. Similar results were obtained when 1,1-dimethyl-4-phenylpiperazinium iodide was used instead of nicotine. Since the action of peptone under these conditions was similar to that of nicotine and DMPP, it is suggested that the effect of peptone on the guinea-pig ileum was due, at least in part, to stimulation of parasympathetic ganglia in the intestinal wall. The gradual return of the response to peptone, 1,1-dimethyl-4-phenylpiperazinum iodide or nicotine after blockade induced by hexamethonium can be explained on the basis of the gradual recovery of the sensitivity of the ganglia following the removal of the antagonist.

The Action of Peptone on the Inferior Eyelid of the Rat

It has been shown by Gertner (1956) that the contraction of the inferior eyelid of the anaesthetized rat provides a sensitive method for studying the effects of drugs acting on smooth muscle and sympathetic ganglia.

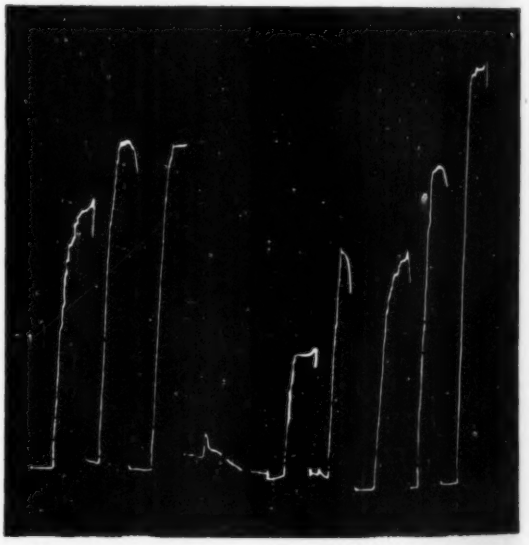

FIG. 3.—Guinea-pig ileum preparation in 10 ml. bath. Effect of 200 μg. hexamethonium (He) (allowed to remain in the bath for 4 min.) on the response to 20 mg. peptone (at P) and 5 μg. nicotine (at Ni). 1 μg. eserine was maintained in the bath throughout the experiment. The substances were added to the bath at 2 min. intervals.

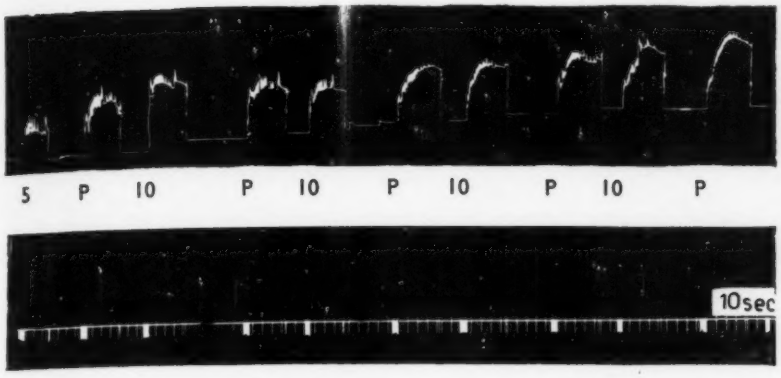

FIG. 4.—Inferior eyelid of rat (400 g.). Chloralose anaesthesia. 5, 10=5 and 10 μg. 1,1-dimethyl-4-phenylpiperazinium iodide. P=5 mg. Witte's peptone. The injections were made into the lingual artery at 10 min. intervals.

Since peptone stimulates sympathetic ganglia in the rat (Beraldo, 1958), it was decided to investigate the effect of peptone on the response of the inferior eyelid of the rat.

When peptone (1.25 to 2.5 mg. or 0.025 to 0.05 ml. of 5% solution/100 g. of body weight) was injected into the lingual artery the inferior eyelid contracted within a few seconds. Repetition of the same dose of peptone at 10 min. intervals produced a constant effect; desensitization or tachyphylaxis did not occur. The effect of peptone was compared with that of 1,1-dimethyl-4-phenylpiperazinium iodide. Thus, the contraction of the inferior eyelid produced by 5 to 10 mg. of peptone in a group of 12 rats was roughly equivalent to that produced by 5 to 50 μg. of 1,1-dimethyl-4-phenylpiperazinium iodide. The result is shown in Fig. 4.

Peptone Response After Hexamethonium.—In order to investigate whether peptone acted directly on the smooth muscle fibre of the inferior eyelid of the rat or through stimulation of sympathetic ganglia, paralysing doses of hexamethonium were used. The effectiveness of ganglionic blockade was tested with 1,1-dimethyl-4-phenylpiperazinium iodide. The response to peptone (2 to 5 mg./100 g. body weight) was followed in a series of 10 rats which had been previously treated with hexamethonium (0.5 to 5 mg.). In 6 of the rats the responses were reduced by 100%; in 2 the reduction lay between 50 and 60%, and in 2 it remained unchanged as compared with the height of the tracing of the contractions of the inferior eyelid immediately before the injection of the hexamethonium. Fig. 5 shows a partial blockade of the effect of peptone and 1,1-dimethyl-4-phenylpiperazinium iodide on the inferior eyelid of the rat as produced by 2.5 mg. hexamethonium. The blockade and recovery of the response to peptone and 1,1-dimethyl-4-phenylpiperazinum iodide followed, as on the guinea-pig ileum, a parallel course suggesting a similar mechanism of action for the two substances.

Effect of Peptone on the Nictitating Membrane of the Cat

The nictitating membrane of the cat did not contract when a dose of 10 to 50 mg. peptone was injected into the lingual artery (3 experiments). However, the injection of 5 to 10 μg. 1,1-dimethyl-4-phenylpiperazinum iodide or nicotine in the same preparation caused strong contractions. Thus, peptone failed to stimulate the superior cervical ganglion of the cat.

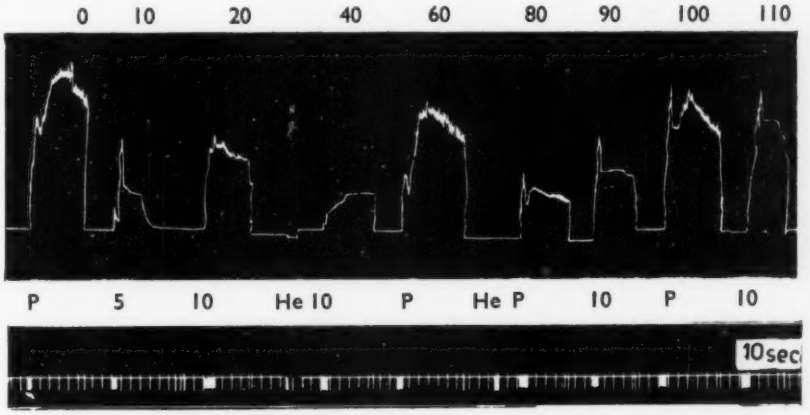

FIG. 5.—Inferior eyelid of rat (370 g.). Chloralose anaesthesia. P=20 mg. Witte's peptone. 5, 10=5 and 10 μg. 1,1-dimethyl-4-phenylpiperazinium iodide. He=intravenous injection of 2.5 mg. hexamethonium. A period of 10 min. elapsed between the injection of hexamethonium and those of 1,1-dimethyl-4-phenylpiperazinium iodide and peptone which were injected into the lingual artery according to time intervals given in min. at the upper part of the tracing.

Some Properties of the Active Principle Present in Peptone

The active principle was dialysable. It was destroyed by boiling in concentrated hydrochloric acid as in the method for extraction of histamine (Code, 1937). However, it withstood boiling for 1 hr. in 0.5 N hydrochloric acid; boiling for 30 min. in sodium hydroxide destroyed it. The treatment of peptone solution with permutit, which removed histamine, did not remove the active principle from the solution. The active principle was partially retained on charcoal, but it was not possible to elute it with 0.05N or 0.1N hydrochloric acid.

The incubation of peptone for 2 hr. with proteolytic enzymes such as chymotrypsin, pepsin and trypsin had no effect on the activity of peptone when tested on guinea-pig gut or inferior eyelid of the rat. Parallel tests were performed with bradykinin and peptone incubated with chymotrypsin. While the activity of the bradykinin was destroyed in 30 min. by chymotrypsin, the activity due to peptone remained unaltered.

Discussion

The results of the experiments described above provide evidence of a ganglion-stimulating principle in peptone. The smooth muscle stimulating effect on the gut was not abolished or diminished when peptone was maintained at *p*H 8.5 for 1 hr. at 20°, whereas equiactive doses of acetylcholine, when submitted to similar treatment, were inactivated. These results indicate that the ganglion-stimulating principle in peptone is unlikely to be acetylcholine or some other ester of choline.

Feldberg (1951) demonstrated that hexamethonium depressed the response of the guinea-pig and rabbit ileum to potassium chloride, and Ambache and Lessin (1955) showed that the response to potassium chloride was consistently reduced after treatment with botulinum toxin. This finding was taken as evidence that part of the action of potassium on the intestine was by stimulation of nervous structures. Both of these authors used relatively large doses of potassium chloride (10 to 15 and 8 to 20 mg., respectively) to evoke contraction of the guinea-pig ileum. Since in our experiments peptone was used in doses ranging from 20 to 40 mg., the action of peptone on the intestine could not have been caused by potassium in the peptone. Further, the effect of peptone disappeared after boiling the solution for 30 min. with N sodium hydroxide or with concentrated hydrochloric acid.

It has been shown that histamine stimulates the superior cervical ganglion of the cat (Konzett, 1952; Trendelenburg, 1954). It was therefore possible that the response of the inferior eyelid to peptone was mediated through the release of histamine. The effect of histamine on the eyelid response was tested in 3 experiments by injecting doses of 0.1 to 1 mg. into the lingual artery. In none of the experiments did the inferior eyelid respond to doses in that range, although a typical response was obtained with peptone. It is interesting to contrast these results with those obtained on the guinea-pig ileum: treatment of this preparation with cocaine abolished the effect of peptone and nicotine, but not that of histamine.

Both the spasmogenic effect of peptone on the guinea-pig ileum and its action on the inferior eyelid of the rat might have been caused by the release of 5-hydroxytryptamine. Robertson (1954a, 1954b) found that 5-hydroxytryptamine stimulated the perfused superior cervical ganglion of the cat and that this action was not blocked by hexamethonium. Rocha e Silva, Valle and Picarelli (1953) obtained evidence that 5-hydroxytryptamine acted on the postganglionic cholinergic fibres of the intramural nervous system of the guinea-pig ileum, which explains why hexamethonium failed to inhibit the action of 5-hydroxytryptamine on this preparation. The spasmogenic action of peptone on the guinea-pig ileum, as well as its action on the inferior eyelid of the rat, however, were diminished or completely inhibited by hexamethonium.

The possible presence in peptone of an active peptide or peptides which might account for its ganglion-stimulating action was investigated. The incubation of peptone with proteolytic enzymes such as chymotrypsin, pepsin and trypsin, however, had no effect on the activity of peptone. These results, however, do not exclude the possibility that the activity is due to a peptide, or peptides, present in peptone.

The negative results obtained with the nictitating membrane of the cat are at variance with those obtained with the inferior eyelid of the rat. The failure of peptone to stimulate the superior cervical ganglion of the cat remains unexplained.

It is possible that peptone formed during protein digestion may be concerned with motility of the gastro-intestinal tract in some animal species.

The authors would like to thank Dr. M. Rocha e Silva (Faculdade de Medicina, Ribeirao Preto) and Eline S. Prado (Escola Paulista de Medicina) for kindly supplying bradykinin and 1,1-dimethyl-4-phenylpiperazinium iodide respectively). This work was supported in part by funds received from the Conselho Nacional de Pesquisas.

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THE ABSORPTION OF PROTHIDIUM BY *TRYPANOSOMA RHODESIENSE*

BY

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(RECEIVED AUGUST 19, 1959)

When rats, heavily infected with *Trypanosoma rhodesiense*, were injected with Prothidium and killed 1 to 5 hours later, measurable amounts of the drug could be extracted from the parasites: a million trypanosomes have been shown to absorb 0.01 to 0.06 μg. of Prothidium *in vivo*. When viewed with the fluorescence microscope, treated trypanosomes appeared to concentrate Prothidium particularly in the blepharoplast and other cytoplasmic granules. Prothidium was absorbed by trypanosomes *in vitro* in less than 30 min. When equilibrium had been reached the concentration of the drug inside the trypanosome was approximately 400 times the concentration outside, in the range of concentrations studied.

Prothidium (7-amino-2-(2-amino-6-methylpyrimidin-4-yl)amino-9-*p*-aminophenylphenanthridine 10,1′-dimethobromide) was introduced by Watkins and Woolfe (1956) as a powerful prophylactic agent against cattle trypanosomiasis. When exposed to ultra-violet light, dilute solutions of the compound in distilled water show a brilliant orange fluorescence with a peak absorption of 315 mμ, which is detectable down to dilutions of 1 μg./ml. This property of fluorescence has been used to study the absorption of Prothidium by *Trypanosoma rhodesiense*.

METHODS

Trypanosomes.—The strain of *T. rhodesiense* used throughout these experiments was the Maun strain which was isolated in December 1955. The parasite, which is polymorphic, produces a fatal infection in hooded Norwegian rats bred in this Institute. It is sensitive to Prothidium; the minimum effective dose in infected mice is 3.75 mg./kg.

Appearance of Trypanosomes after Treatment with Prothidium.—A fresh blood film was taken from the tail of a heavily infected rat 1 hr. after an intraperitoneal dose of 7.5 mg./kg. of Prothidium. The coverslip was sealed to the slide with a Vaseline-paraffin wax mixture and the preparation was examined with the fluorescence microscope (Fig. 1). The fluorescence microscope was a simple arrangement of components; no costly apparatus was used.

Extraction and Estimation of Prothidium.—Intraperitoneal doses of 7.5 mg./kg. of Prothidium were given to 8 rats, all of which had 40 or more trypanosomes in each low-power field of a fresh blood preparation. The animals were killed 4.5 to 5 hr later and the blood collected and heparinized.

The trypanosomes were separated by differential centrifugation, washed three times in saline and then counted; they were still actively motile at this stage. The trypanosomes were spun down, the saline was removed and replaced by 4 ml. of 5N HCl in order to extract the drug from the parasites. Five min later, the debris was removed by centrifugation and the supernatant examined under ultra-violet light. Untreated trypanosomes were used as a control and were similarly extracted. The acid extract from treated trypanosomes fluoresced yellow in the ultra-violet whereas the controls did not fluoresce at all. Prothidium fluoresces yellow in acid solution and orange at *p*H 7.0 to 8.5. The fluorescence is quenched at *p*H 9.0 and over; the reactions are reversible. The Prothidium was estimated quantitatively by determining the optical density of the acid extract in a Beckman spectrophotometer (the control extract serving as a blank), using the wavelength 315 mμ. The U.V. absorption curve for Prothidium in 5N HCl is shown in Fig. 2. A standard curve prepared at 315 mμ is shown in Fig. 3.

Absorption of Prothidium by the Trypanosome in Vitro.—Solutions of Prothidium in saline were prepared so that 0.5 ml. samples contained 20 to 80 μg. of Prothidium and these samples were placed in a series of small glass tubes of 0.5 cm. bore. A concentrated suspension of trypanosomes was obtained by centrifuging the blood of a heavily infected rat. These were washed in nutrient medium (1 part of horse serum with 3 parts of Ringer solution containing 0.2% of glucose) and then suspended in this medium so that there were 140 to 280 million trypanosomes

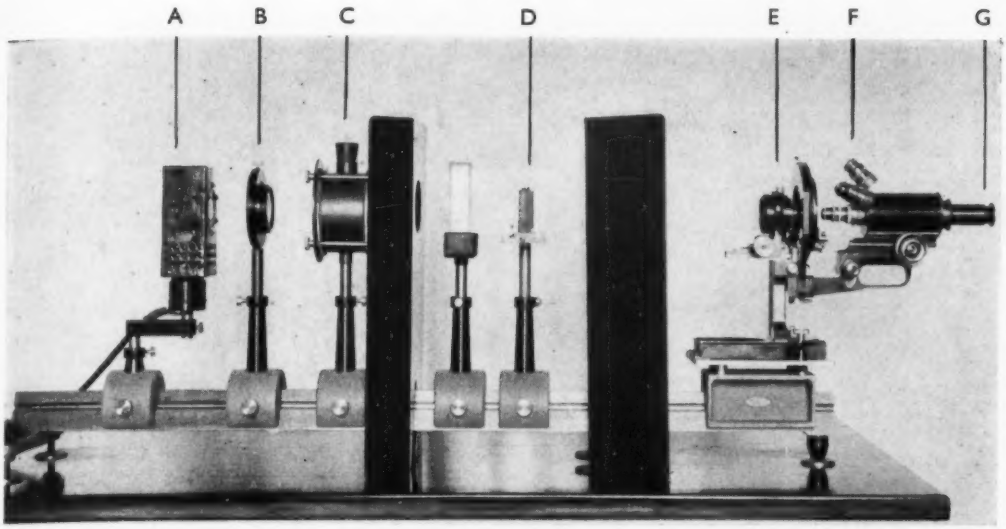

FIG. 1.—The fluorescence microscope. **A**, Activating light source (box type mercury vapour lamp). **B**, Collecting lens. **C**, Heat absorber. **D**, Wratten 18A primary filter. **E**, Cardioid condenser. **F**, Apochromatic objectives (2 mm. 1.4 NA used in this work). **G**, Ocular × 6 (no secondary filter).

ml. Samples of this suspension measuring 0.5 ml. were then placed in each of the small tubes containing Prothidium and incubated for 30 min. at 35°. Control tubes containing Prothidium and nutrient medium alone were also set up. After incubation the trypanosomes were spun down by centrifugation (800 *g*) and the supernatant of each tube was removed into 3 ml. of 5N HCl and the Prothidium content determined. Thus the amount of the drug absorbed by the trypanosomes could be calculated. The partition ratio (concentration inside trypanosomes/concentration outside) was calculated by the method of Hawking (1944). The amount of Prothidium in the trypanosomes was given by the amount which had disappeared from the supernatant; the volume of 100 million trypanosomes was taken to be 2.4 mm.[3]

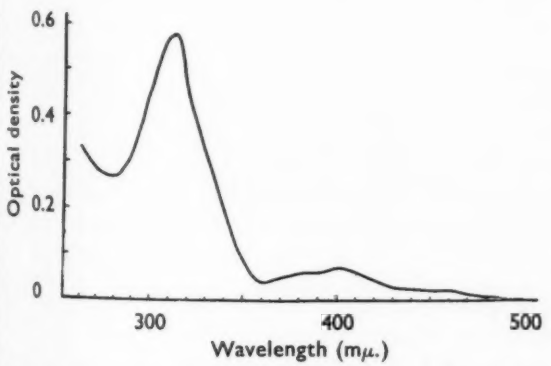

FIG. 2.—The ultra-violet absorption curve of Prothidium in 5N HCl.

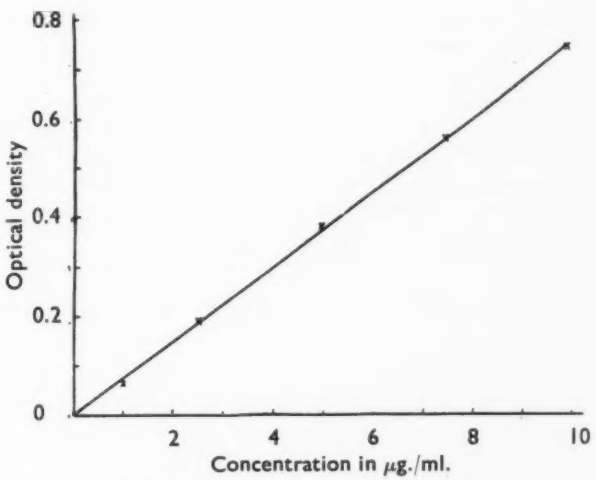

FIG. 3.—Standard curve showing the ultra-violet absorption of Prothidium in 5N HCl at various concentrations, using the wavelength 315 mμ.

RESULTS

Trypanosomes from rats treated with Prothidium exhibited a brilliant orange fluorescence (Fig. 4). The intensity of the fluorescence varied with the type of trypanosome. In the stumpy forms the blepharoplast, the nucleus and the cytoplasmic granules around it shone brilliantly. Some granules in the anterior region of the trypanosome also fluoresced brightly; they

FIG. 4.—*Trypanosoma rhodesiense* after treatment with Prothidium *in vivo*. × 2,750.

were probably volutin granules. The cytoplasm also fluoresced but the flagellum was invisible. The red cells and plasma were non-fluorescent. Long thin trypanosomes did not show much fluorescence in the cytoplasm or nucleus, but their cytoplasmic granules and blepharoplasts shone brilliantly. Thus both types of trypanosome had a strong affinity for Prothidium, which was absorbed and deposited in the blepharoplast and other cytoplasmic granules.

Absorption of Prothidium by Trypanosomes.—Table I shows a million trypanosomes absorbed between 0.01 and 0.06 μg. of Prothidium from the blood of a rat 4.5 to 5 hr. after intraperitoneal injection. When trypanosomes were exposed to the drug *in vitro*, equilibrium was reached in 15 to 30 min. After 30 min. the organisms were still actively mobile, but had absorbed a considerable proportion of the Prothidium originally present (Table II). Fig. 5 shows the partition of Prothidium between trypanosomes and medium. The partition ratio diminished with increasing concentrations of Prothidium. In Fig. 5, the mean concentrations of drug have been calculated from the data in Table II. In these experiments there was a tendency for the curve to flatten off at the higher concentrations; even at the lower concentrations the slope did not reach 45°. Over the range of concentrations of drug used (10 to 45 μg./ml.) the partition ratio ranged from 140 to 590. For the purpose of comparing Prothidium with other drugs a mean figure for the partition ratio may be taken as 400.

TABLE I

THE AMOUNTS OF PROTHIDIUM RECOVERED FROM THE TRYPANOSOMES OF RATS HEAVILY INFECTED WITH *T. RHODESIENSE*

7.5 mg./kg. Prothidium was given intraperitoneally.

Hr. after Dose	Number of Trypanosomes Extracted × 10^6	Total Drug Extracted in μg.	Drug Absorbed by 10^6 Trypanosomes in μg.
4·5	323	20·4	0·063
4·75	307	12·52	0·041
	280	17·2	0·062
	247	6·4	0·025
	272	32·0	0·012
	142	6·4	0·044
	102	31·0	0·031
5·0	178	10·0	0·056

DISCUSSION

The above experiments have demonstrated that "fixation" of Prothidium by *T. rhodesiense* occurs in less than 1 hr. both *in vivo* and *in vitro*.

It has also been shown that there is a quantitative relation at equilibrium between the concentration of Prothidium inside the trypanosomes and that outside; the partition ratio over the range of concentrations studied was approximately 400. The partition ratio for stilbamidine

ERRATUM

In a paper by R. V. Brown in the March 1960 issue, Fig. 2 on p. 172 was printed upside down. The figure is correctly reproduced here for the convenience of readers who may wish to paste it into their copies.

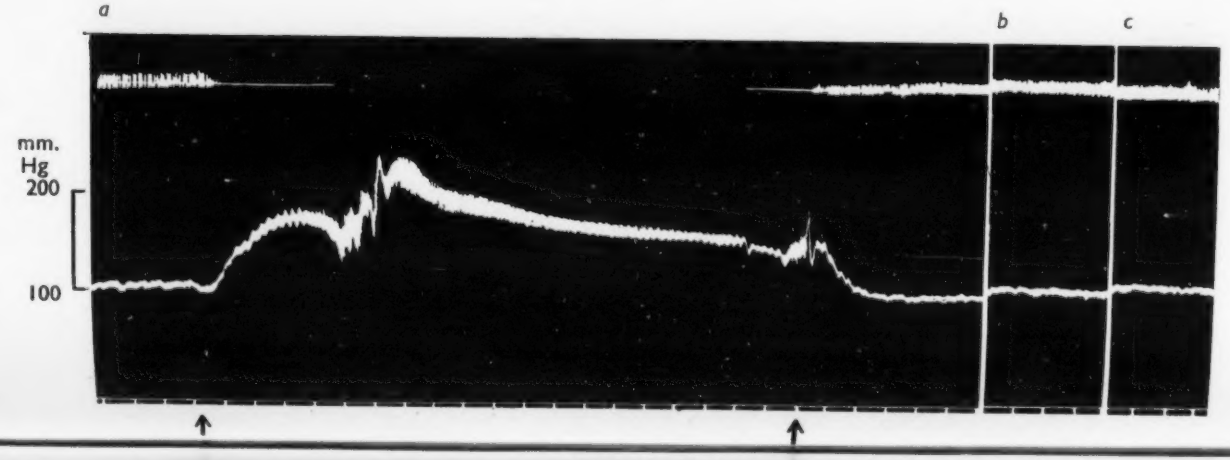

TABLE II

THE ABSORPTION OF PROTHIDIUM BY *T. RHODESIENSE* AFTER EQUILIBRIUM HAS BEEN REACHED *IN VITRO*

Temperature 35°; duration of exposure 30 min.

Initial Concentration of Drug: μg./ml.	Trypanosomes/ml. × 10^6	Final Concentration of Drug: μg./ml.		Partition Ratio	Drug Absorbed by Trypanosomes: μg. (Mean in Brackets)
		Supernatant Fluid	Trypanosomes		
43·6	71·3	30·8	7,500	200	12·8
		28·4	10,100	355	16·4
		34·8	4,900	140	8·85 (12·3)
		31·6	7,000	220	12·1
28·8	71·3	17·6	6,600	375	11·3
		16·6	7,200	435	12·3
		18·7	6,000	410	10·4 (11·3)
		17·6	6,600	375	11·3
12·4	71·3	5·4	3,150	580	6·4
		10·0	5,900	590	2·4
		8·4	4,950	590	4·1 (4·5)
		7·2	4,200	585	5·3
42·8	140	33·6	3,600	107	9·1 (9·7)
		32·4	3,100	96	10·25
25·8	140	18·7	2,120	114	7·25 (6·6)
		20·0	1,940	97	5·85
10·2	140	9·2	2,950	320	0·8 (1·3)
		8·4	3,200	380	1·8

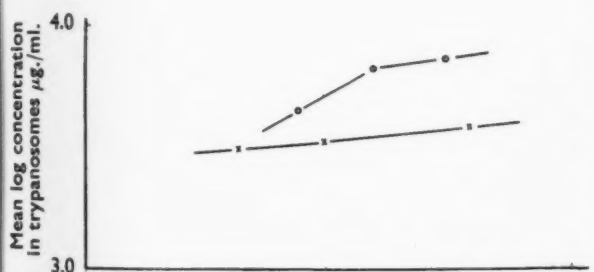

FIG. 5.—The relationship between the concentration of Prothidium in the medium and the concentration in the trypanosomes after equilibrium has been reached at 35° (30 min. exposure).

is about 1,100, for reduced tryparsamide about 5,000 and for acriflavine is about 8,000 (Hawking, 1938 and 1944) so that Prothidium was absorbed less avidly than these three compounds. Examination, by the fluorescence microscope, of trypanosomes which have absorbed the drug shows that it is not evenly distributed over the surface of the cell, but that it is concentrated into the blepharoplast and certain other granules. In this respect, the absorption of Prothidium by trypanosomes is similar to the absorption of stilbamidine (Hawking and Smiles, 1941; Hawking, 1944). The behaviour of stilbamidine is closely similar to that of trivalent arsenicals and acriflavine (Yorke, Murgatroyd and Hawking, 1931; Hawking, 1938) although studies of drug resistance indicate that stilbamidine is absorbed by a different receptor (Lourie and Yorke, 1938; Fulton and Grant, 1955).

Histochemical studies have led Ormerod (1951) to suggest that phenanthridinium compounds act by splitting cytoplasmic ribonucleoprotein into its constituent nucleic acid and protein. Newton (1957) has shown that Promidium (Ethidium bromide) rapidly inhibits deoxyribonucleic acid synthesis in *Strigomonas oncopelti* whilst ribonucleic acid and protein synthesis is apparently unaffected. Since Prothidium is also a phenanthridinium compound, it may be that it exerts a similar action on *T. rhodesiense*.

My grateful thanks are due to Dr. F. Hawking for his advice and encouragement and to Messrs. Boots Pure Drug Company for supplying the Prothidium. I would also like to thank Miss B. C. Staehelin for her willing and skilful technical assistance and Mr. M. Young for taking the photographs.

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THE ABSORPTION, DISTRIBUTION AND EXCRETION OF PROTHIDIUM IN RATS, RABBITS AND CATTLE

BY

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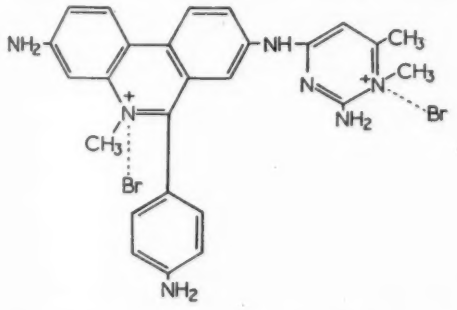
From the National Institute for Medical Research, Mill Hill, London

(RECEIVED AUGUST 19, 1959)

2-Amino-7-(2-amino-6-methylpyrimidin-4-ylamino)-9-*p*-aminophenylphenanthridine 10,1′-dimethobromide (Prothidium), a prophylactic drug against cattle trypanosomiasis, was concentrated in the liver and kidneys of rats and rabbits after intraperitoneal or intracardial injection; it was detectable in these organs for 7 days in rats and 10 days in rabbits. The drug protected adult rats against *Trypanosoma vivax* for 8 weeks. Histological examination of the organs of rats treated with Prothidium indicated that no damage had been incurred from the treatment. When cattle were treated subcutaneously, elongated swellings appeared at the site of injection which disappeared within 6 weeks. Excretion of unchanged Prothidium occurred *via* the bile in rats and the drug was detectable in the bile for 9 days, but no Prothidium could be detected in the faeces or urine of rats or rabbits. No metabolic products of the Prothidium were found in the tissues or plasma of rats, rabbits, or cattle. In rat liver perfused for 6 hours with Prothidium only the unchanged drug was recovered. A depot of Prothidium was formed at the site of subcutaneous injection in cattle and this remained for at least 3 months. The prolonged prophylactic action was probably due to the formation of this depot since Prothidium injected intraperitoneally into a calf was excreted at a similar rate to that observed in rats and rabbits.

Prothidium (2-amino-7-(2-amino-6-methylpyrimidin-4-ylamino)-9-*p*-aminophenylphenanthridine 10,1′-dimethobromide) was introduced by Watkins and Woolfe (1956) as a powerful prophylactic agent against cattle trypanosomiasis.



An earlier paper (Taylor, 1960) has described the absorption of Prothidium by *Trypanosoma rhodesiense*. The present studies are concerned with the absorption, distribution and excretion of Prothidium when administered to rats, rabbits and cattle. The effectiveness of the drug as a prophylactic agent against *T. vivax* in rats was also investigated.

METHODS

Prothidium is an orange powder, readily soluble in water or acid. It is less soluble in alcohol and other organic solvents. Solutions are strongly fluorescent, the colour depending on *p*H: acid solutions fluoresce yellow, neutral solutions fluoresce orange, and the fluorescence is quenched by strong alkali. A specially purified sample of Prothidium (kindly supplied by Dr. G. Woolfe of Messrs. Boots Pure Drug Company, Nottingham) was used in all experiments with the exception of those in cattle, where commercial Prothidium was used.

The free base may be extracted into butanol from alkaline solution and quantitatively back-extracted into N sulphuric acid. The solution in sulphuric acid has a characteristic ultra-violet absorption spectrum (maximum at 315 mμ, Fig. 1) and this has been used throughout to identify the drug in tissue extracts. For the estimation of Prothidium at low concentrations it was more convenient to use the more sensitive fluorimetric method described below.

Chemical Methods

Extraction of Prothidium from Tissues.—Preliminary evidence suggested that Prothidium was strongly bound to proteins; dialysis of solutions of Prothidium in plasma or in serum at 4° against

normal saline extracted only 50% of the drug. When a solution of Prothidium in water was dialysed in a similar manner, almost all the drug passed into the dialysate.

Good recovery of Prothidium from either tissue or from plasma was obtained when the material (5 g.) was homogenized with 2N sulphuric acid (30 ml.) in an all-glass Potter homogenizer. The resulting suspension was centrifuged at 16,000 *g* at 4° for 40 min., and the solids were discarded. The supernatant fluid was extracted with ether (10 ml.) to remove fats and subsequently made alkaline by the addition of sufficient 40% sodium hydroxide (7 ml.) to neutralize the sulphuric acid. The Prothidium base was extracted into butanol (10 ml.) and centrifuged to facilitate separation.

The loss on extraction was small and was accepted as a necessary defect of a simple routine method for use on large numbers of samples. For liver, the loss, estimated by recovery of Prothidium (100 μg.) added to a liver homogenate (5 g.) and extracted under the standard conditions described above, was 25 μg. (25%). Recovery from plasma (5 ml.) was 95%. No correction factor has been applied in the estimation of recovery in actual experiments since the percentage loss varies somewhat with different tissues and fluids. The distribution in different organs is unaffected by these losses.

Estimation of Prothidium by Fluorescence.—The butanol extract was washed once with an equal volume of distilled water and dried over anhydrous potassium carbonate (2.5 g.) for 30 min. Fluorescence of the dried butanol extract was measured using a Farrand fluorimeter with an orange secondary filter (Wratten No. 25). A blank was obtained by extracting normal tissue in a similar manner. The concentration of Prothidium present was estimated by comparison with a standard curve (Fig. 2). The performance of the fluorimeter was standardized using aqueous eosin solution at a final concentration of 50 μg./ml. As a check that the fluorescence was due to Prothidium, a sample of the butanol extract was mixed with N sulphuric acid and separated by centrifugation. The ultra-violet spectrum of the sulphuric acid extract was measured in a Beckman spectrophotometer; the peak of the absorption spectrum curve (260 mμ–500 mμ) served to characterize the drug (Fig. 1).

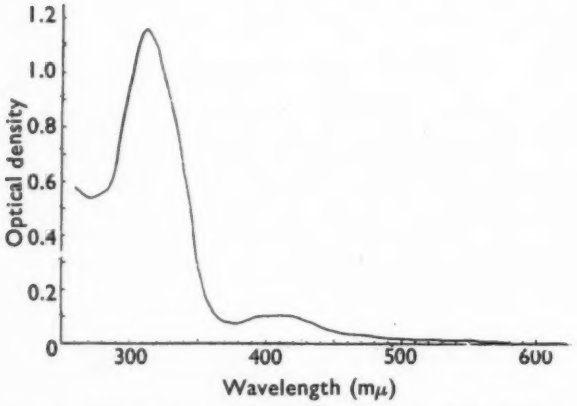


FIG. 1.—The ultra-violet absorption curve of Prothidium in N sulphuric acid.

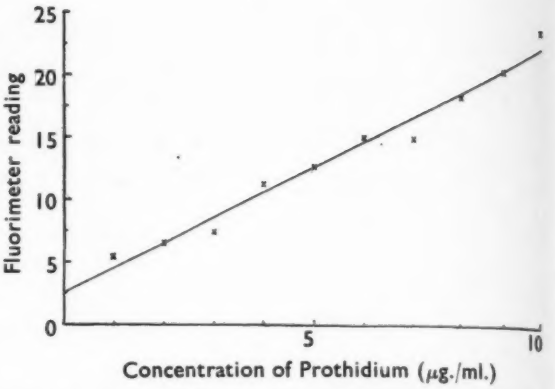


FIG. 2.—The calibration curve of Prothidium for the Farrand fluorimeter.

Biological Methods

Tissue Concentrations.—Prothidium was injected into adult rats and rabbits intraperitoneally (7.5 mg./kg.) or intracardially (3.7 mg./kg.). Groups of 2 or 3 animals were killed at intervals and their tissues assayed for Prothidium.

Excretion.—Thirty adult rats, in 10 groups of three, were used in these experiments. Two rats in each group were injected intraperitoneally with Prothidium and the third rat reserved as a control. Immediately after the dose, the bile from the rats in the first group was collected through a cannula in the bile duct over a 24 hr. period, after which the rats were killed. Thereafter, each day for 9 days, a fresh group of rats was treated similarly. The abdomen was opened under ether anaesthesia; the bile duct was identified and freed from the lesser omentum along one inch of its length. The duct was then cannulated using polythene tubing (0.1 cm. diameter) which had been introduced through the side wall of the abdomen. The abdomen was closed and the rat maintained for 24 hr. in a restraining cage whilst the bile was collected. The Prothidium content of the bile (15 to 20 ml.) was estimated after the addition of an equal volume of 2N sulphuric acid and extraction in the usual way.

Perfusion of Rat Liver with Prothidium.—Adult rats were starved overnight and 5 to 8 ml. of blood were removed by cardiac puncture under ether anaesthesia. About 55 ml. of blood was collected in siliconed glass tubes for each perfusion. The plasma was separated from the erythrocytes by centrifugation.

To this plasma was added 30 mg. of powdered Prothidium and the solution continuously stirred using a glass rod. Ten minutes later a clot had formed on the rod; the volume of the clot was decreased by rolling it around the inside of the tube for about 5 min. The plasma was then centrifuged at about 600 *g* for 2 min.; 1 ml. was removed for fluorimetric estimation of Prothidium, and the *p*H of the remainder adjusted to 7.4 with sodium bicarbonate. The treated plasma was then remixed with the erythrocytes, which had been washed once with saline to remove any traces of plasma, and the whole used as the liver perfusate.

The technique of liver perfusion used was that described by Cohen and Gordon (1958), and the surgical technique and the perfusion apparatus were basically as described by Miller, Bly, Watson, and Bale (1952). The liver donor (hooded male rat) was starved overnight. The abdomen was opened along the mid-line, the bile duct and the portal vein identified and cannulated without previous ligation of the gastric and duodenal blood vessels. Perfusion of the liver was commenced as soon as the superior vena cava had been cannulated; the liver was then removed from the animal. Initially, the liver was perfused with Ringer solution to wash out any traces of the donor blood, and the Ringer was then replaced with the treated blood described above. The perfusion was allowed to run for 5 hr., and during this time the bile was collected.

At the end of the experiment the blood was collected for extraction of Prothidium. A 3 mm. cube of liver was frozen in dry ice for histological sectioning at −20°; a 5 mm. cube of liver was fixed in Carnoy and another fixed in formal saline, both for histological sectioning. The remainder of the liver was homogenized in 2N sulphuric acid and the Prothidium was extracted and determined. In some experiments, plasma and bile were extracted by various methods in an attempt to detect any metabolites. Samples were also subjected to paper electrophoresis and chromatography.

A homogenate of the perfused liver was subjected to electrodialysis using the method of Molle (1956). An acid liver brei (8 g. liver in 30 ml. 0.1 N sulphuric acid) was placed in the central cell and the two outer cells were filled with water. Platinum electrodes were used and 100 mA. was passed through the apparatus until the current had fallen to a steady minimum (after about 2 hr.) indicating that all the ions present had travelled to the electrodes. The contents of the cathode cell were removed into 2 ml. of 40% sodium hydroxide, extracted with 10 ml. butanol and back-extracted into N sulphuric acid, and the ultra-violet absorption spectrum determined.

Distribution of Prothidium in a Calf.—Liver biopsies were carried out on a 6-months calf. I am indebted to Mr. Ford of the A.R.C. Unit, Babraham Hall, Cambridge, who kindly demonstrated the technique of liver biopsy. The method used was that described by Loosmore and Allcroft (1951). The tissue samples thus obtained were homogenized in acid and assayed for Prothidium in the usual way. The first liver biopsy, taken 3 days after intraperitoneal injection of 140 mg. of Prothidium in 7 ml. water, yielded 650 mg. of liver. A second liver biopsy (700 mg.) was taken 18 days after injection. Thirteen days later (1 month after injection) the calf was slaughtered and the liver, kidney, and a sample of bile were removed. Samples of the liver, kidney, spleen, muscle, lung, and heart were fixed in formal saline and examined histologically.

Absorption in Cattle.—Three adult barren Ayrshire cows were each given a subcutaneous injection of a 4% solution of Prothidium in water (2 mg./kg.). The site of injection was about a 6-in. square over the eleventh and twelfth ribs about 8 to 10 in. down from the dorsal mid-line.

Prophylactic Activity Against T. vivax *in Rats.*—Adult or young (60 to 80 g.) hooded rats bred in this Institute were given 7.5 mg./kg. of Prothidium by intraperitoneal injection. This dose is close to the toxic range. A group of 3 treated rats, and 2 control rats, were infected immediately with 8 to 12m. parasites. Further groups were inoculated at weekly intervals. Tail blood films were taken daily for six

TABLE I

THE DISTRIBUTION OF PROTHIDIUM AFTER INTRAPERITONEAL AND INTRACARDIAL INJECTION INTO RATS

Route	Intervals after Injection	Percentage of Dose Recovered (Mean Values in Brackets)	
		Liver	Kidneys
Intraperitoneal	2 hr.	11, 26, 24 (20·1)	
	6 ,,	21, 15, 17 (17·5)	
	9 ,,	16, 25, 21 (20·5)	
	12 ,,	18, 23, 25 (21·8)	
	24 ,,	30, 28, 38 (29·0)	5, 2 (3·45)
	48 ,,	16, 9, 11 (11·5)	4, 2 (3·0)
	72 ,,	11, 5, 11 (8·7)	3, 2 (2·5)
	96 ,,	5, 7, 1 (5·3)	0·5, 3 (1·75)
	120 ,,	Trace	3, 3 (3·0)
	192 ,,	0	3, 2 (2·5)
	216 ,,	0	2, 1 (1·5)
Intracardial	2 ,,	23, 15, 12 (16·6)	
	6 ,,	21, 18, 20 (19·5)	
	9 ,,	26, 42, 29 (32·2)	
	12 ,,	37, 48, 27 (36·2)	
	24 ,,	26, 29 (31·0)	
	48 ,,	22, 15 (22·0)	
	72 ,,	3, 10 (3·2)	
	96 ,,	7, 4 (5·4)	
	120 ,,	Trace	
	192 ,,	0	
	216 ,,	0	

days after infection and thereafter weekly for 7 to 11 weeks, and examined for the presence of trypanosomes. The strain of *T. vivax* in this work was the Ilorin (unsupplemented line) strain described by Desowitz and Watson (1953); this strain was made available to me by the courtesy of Mr. Reed of the Wellcome Laboratories of Tropical Medicine, London. The parasite produces a severe and usually lethal infection in laboratory rats and mice.

Results

Absorption and Excretion in Rats and Rabbits

The fluorescence of Prothidium was used to follow visually the fate of the drug after injection. Freshly killed rats were observed under an ultra-violet lamp 2 to 6 hr. after intraperitoneal injection of Prothidium. Under these conditions the liver and kidneys took on an orange fluorescence; the xiphisternum and a small area of the abdominal wall around the site of injection also fluoresced. No orange fluorescence was observed elsewhere in the animals.

The percentages of the dose recovered from rat liver after intraperitoneal and intracardial injections and from rat kidneys after intraperitoneal injections are recorded in Table I; the rates of disappearance of Prothidium from the liver and kidneys of rats and rabbits are shown in Figs. 3 and 4. There were considerable differences between individual animals, but it is clear that the drug appeared in considerable quantities in the liver and kidneys quite soon after injection. Little or no drug was detectable in the gut, spleen, heart, lung or muscle of rats. The rate of disappearance of the drug from the liver and kidneys of both rats and rabbits was rapid for the first 24 hr. after injection, but the drug was detectable in these organs for 7 days in rats and for 10 days in rabbits. A typical ultra-violet absorption curve of these extracts is shown in Fig. 5, demonstrating that unchanged Prothidium was present in the tissues.

Histological sections of treated liver (cut at $-20°$), when viewed with the fluorescence microscope, showed a concentration of fluorescent material in the nucleoli, nuclear membrane and basophilic granules of the hepatic cells. The Kupffer cells were loaded with fluorescent

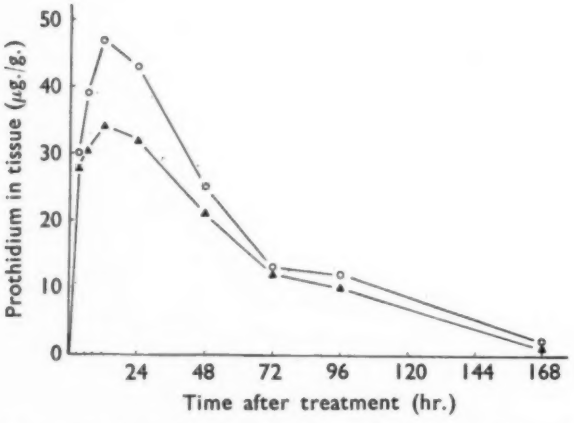


FIG. 3.—The rate of elimination of Prothidium by rat liver and kidneys. O—O = liver (each point on the curve represents the average of 6 rats; 3 rats were injected intraperitoneally and 3 were injected intracardially, but, as the results from each route were similar, the average of the 6 rats was used). ▲—▲ = kidneys (each point on the curve represents the average of 3 rats after intraperitoneal injection).

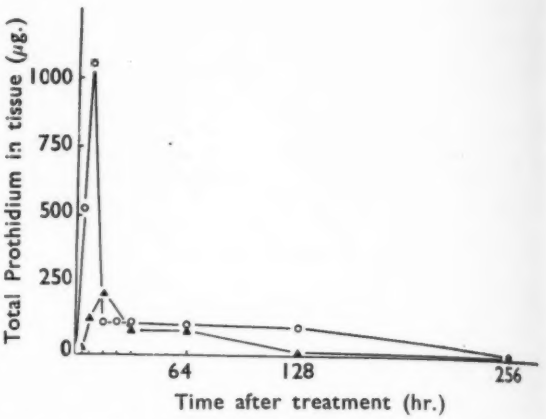


FIG. 4.—The rate of elimination of Prothidium from rabbit liver and kidneys. Each point on the curve represents the average of 2 rabbits, after intraperitoneal injection. O—O = liver. ▲—▲ = kidneys.

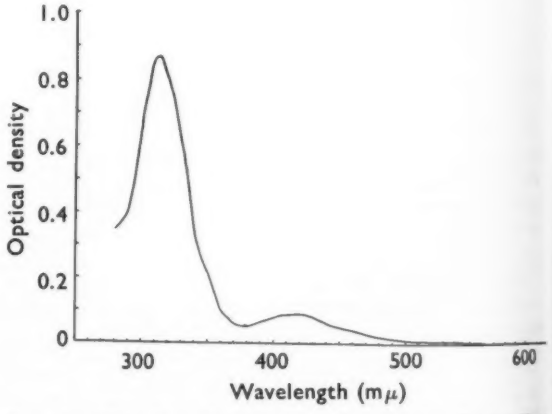


FIG. 5.—The ultra-violet absorption curve of a rat liver extract in N sulphuric acid. The liver of a treated rat was homogenized in 2N sulphuric acid, extracted into butanol and back-extracted into acid.

TABLE II

EXCRETION OF PROTHIDIUM IN THE BILE OF RATS

Each result represents the amount of Prothidium extracted from the bile excreted by one rat in 24 hr. One animal was used for each time point.

Time after Injection (hr.)	Prothidium in Bile (μg.)	Prothidium Given (μg.)	Percentage of Prothidium Recovered/ 24 hr.	Mean Percentage Recovery/ 24 hr.
0–24	135·3 209·0	2,000 1,900	6·75 11	8·9
24–48	285·0 104·0	1,500 2,300	19 4·5	11·7
48–72	46·0 137·0	1,600 2,500	2·85 7·0	4·9
72–96	39·6 43·0	1,800 1,900	2·20 2·25	2·2
96–120	25·6 25·0	1,600 1,550	1·6 1·6	1·6
120–192	24·0 24·2	2,200 2,200	1·1 1·1	1·1
192–216	8·64 10·0	1,600 2,300	0·54 0·44	0·5
	Total Prothidium recovered (mean of each 2 rats)= 558·2 μg.	Average μg. of Prothidium given =1,920 μg.	Percentage of Prothidium recovered in 216 hr.= 30·9%	

material and were frequently swollen with it. Sections of treated liver stained with haematoxylin and eosin appeared little damaged by the drug.

Excretion

Urine from rats and rabbits which had received Prothidium contained little if any of the drug. No Prothidium could be detected by heating the urine samples with acid or alkali to hydrolyse any metabolic derivative, by electrophoresis, by continuous ether extraction or by paper chromatography. If any Prothidium is excreted by this route it can only be a very small amount.

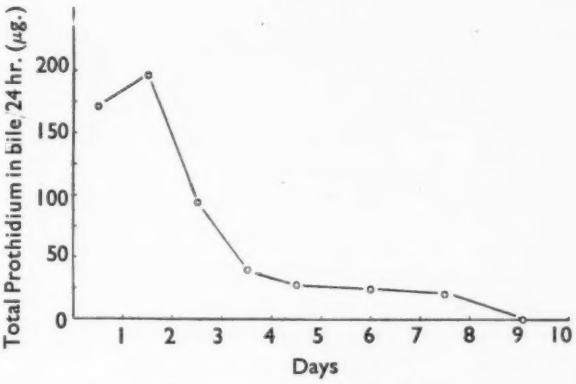


FIG. 6.—The rate of elimination of Prothidium in rat bile. Each point on the curve represents the average of 2 rats.

Since the liver was heavily loaded with Prothidium and since excretion of the drug in the urine appeared to be negligible, the possibility was considered that secretion took place via the bile.

Table II shows the results of experiments in which bile was collected from treated rats; the rate of elimination of Prothidium from the bile is shown in Fig. 6. The drug was rapidly excreted in the bile for the first 3 days and was detectable for 9 days. About 31% of the initial dose of Prothidium was recovered from the excreted bile in 9 days. The ultra-violet absorption curves of the bile extracts corresponded closely with that of the pure drug.

Absorption by Perfused Rat Liver

In view of the concentration of Prothidium in the liver and its rapid elimination from this organ, it was considered possible that this was the site of metabolism of the drug. Plasma, bile and liver tissue taken after 5 hr. of perfusion and examined by various methods of extraction and by paper electrophoresis and chromatography failed to yield evidence of metabolites of Prothidium. A butanol extract of the cathode cell after electrodialysis of a homogenate of perfused liver gave an ultra-violet absorption spectrum which corresponded closely with that of pure Prothidium; no trace of metabolic products could be found.

Free Prothidium was also found in the plasma perfusate and in the bile, but no metabolic product could be detected. Almost all the Prothidium used in the liver perfusion was recovered at the end of the experiment.

TABLE III

THE DISTRIBUTION OF PROTHIDIUM IN CATTLE AFTER INTRAPERITONEAL OR SUBCUTANEOUS INJECTION

i.p.=intraperitoneal; s.c.=subcutaneous.

Animal	Prothidium Given (mg.)	Route	Time after Treatment	Sample	Wt. or Vol. Sample	Conc. of Prothidium in Sample	Total Prothidium Recovered(μg.)	Fluorescence
Calf	140	i.p.	3 days	Liver	0·65 g.	5 μg./0·1 g.	35	
			18 ,,	,,	0·7 ,,	Trace	Trace	
			31 ,,	,,	3·1 kg.	,,	,,	Yellow
				Kidney	660 g.	Nil	Nil	Nil
				Bile	9 ml.	Trace	Trace	
				Muscle	Section			,,
				Lung	,,			,,
				Heart	,,			,,
Cow I (4–5 yr.)	1,026	s.c.	3 weeks	s.c. tissue	28·3 g.	35 μg./g.	994	
				,, fluid	12 ml.	20 μg./ml.	240	
,, II (4–5 yr.)	988	,,	3 ,,	,, ,,	23 ,,	9·9 ,,	227	
,, III (2–3 ,,)	978	,,	3 ,,	,, ,,	31·5 ,,	10·1 ,,	318·2	
,, II			6 ,,	,, tissue	18·2 g.	33 μg./g.	600·6	
,, III			12 ,,	,, ,,	17·3 ,,	15·4 ,,	267	

The Absorption of Prothidium in Cattle

The results of experiments in cattle treated with Prothidium are shown in Table III. The first liver biopsy, taken 3 days after injection of the drug in a calf, contained Prothidium, but no trace of the drug was found in a second specimen taken 18 days after injection. When the calf was slaughtered one month after injection, traces of Prothidium were present in the bile and liver but none was found in the kidneys. The histological sections of all the tissues examined appeared normal; only the (unstained) liver sections showed a yellow fluorescence when viewed with ultra-violet light.

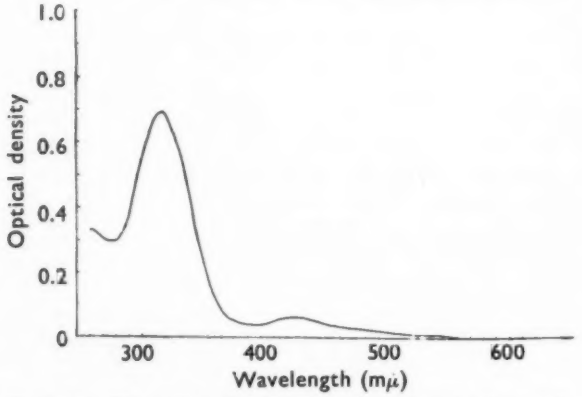


FIG. 7.—The ultra-violet absorption curve of a subcutaneous tissue extract in N sulphuric acid from cow 1. The subcutaneous tissue was homogenized in 2N sulphuric acid, extracted into butanol and back-extracted into acid.

When Prothidium was injected subcutaneously into cows, elongated swellings appeared at the site of injection in all 3 animals; the swellings remained for 6 weeks. They contained a fluid exudate with a high concentration of Prothidium 3 weeks after injection. The subcutaneous tissue at the site of injection also contained a high concentration of drug (Table III; Fig. 7). Six weeks and 12 weeks after injection the subcutaneous tissue at the injection site still contained much unchanged Prothidium, but the swelling had disappeared and there was no fluid.

Prophylactic Action in Rats

A single dose of 7.5 mg./kg. of Prothidium protected rats against *T. vivax* for about 8 weeks and young rats for at least 5 weeks. Young rats developed a transient infection before becoming permanently cured; infected control rats died 2 to 4 days after inoculation. Adult rats were more resistant to infection and untreated infected animals sometimes survived.

DISCUSSION

A single dose of Prothidium will protect cattle against trypanosomiasis for six months (Watkins and Woolfe, 1956). Suramin (Bayer 205) has a

similar prophylactic action in human trypanosomiasis and in this instance the prolonged protection is due to retention of the drug. This retention has been demonstrated for the rat, guinea-pig and monkey (Findlay, 1930). It seemed likely therefore that the protection afforded by Prothidium might also be due to the retention of the drug or its metabolites.

In the present investigation the method used for the estimation of Prothidium was capable of detecting the drug, added to plasma, down to a level of about 2 μg./ml. This is the same order of sensitivity as that of the method used by Boursnell and Wormall (1939) for the estimation of suramin. In contrast to their results with suramin, Prothidium was not detectable in rat blood 1 hr. after intraperitoneal injection of the drug. However, the Prothidium was strongly bound to liver proteins so that the blood concentration was not an adequate measure of the amount of drug retained in the body. When rats were given Prothidium either intraperitoneally or intracardially, the drug could be detected in liver and kidneys for 7 days, and significant levels of Prothidium were detected in the bile secreted between the 7th and 8th days. From a series of experiments in which bile was collected from rats over 24 hr. periods, it was evident that the bile was a major route for the excretion of Prothidium. Examination of the faeces yielded no evidence of the presence of the drug or its metabolites. Prothidium may be broken down by the gut flora or it may be reabsorbed by the small intestine. The concentration of Prothidium in rat's liver and kidney and its excretion *via* the bile show that it is similar in action to another phenanthridinium compound, carbidium ethanesulphonate, which has been shown to have a similar distribution in mice (Goodwin, Goss, and Lock, 1950).

The results of the distribution and excretion experiments in rats suggested that Prothidium would protect these animals against *T. vivax* infection for at least 10 days. Experiments have shown that a single dose of Prothidium protected adult and young rats against *T. vivax* for about 7 weeks in spite of the fact that the young rats developed a transient infection before being fully protected. It may well be that sufficient Prothidium remained bound within the rat tissues to afford protection for this period of time, but there seemed a disinct possibility that Prothidium was converted to an active metabolite which was responsible for the prolonged action of the drug. No metabolite was detected in urine, in blood or in extracts of a liver which had been perfused *in vitro* with the drug, but this evidence is inconclusive because low concentrations of unknown metabolites would almost certainly have escaped detection. In order to obtain significant results on the excretion of metabolites it would be necessary to use radioactive Prothidium and this has not, so far, been available.

The more prolonged prophylactic action of Prothidium in cattle suggested that there might be marked species differences in the rate of excretion of the drug, and accordingly some of these experiments were repeated using rabbits. In these animals Prothidium was detectable in liver and in kidneys for 10 days after a single intraperitoneal injection ; the drug also disappeared at about the same rate from the tissues of a calf after it had received an intraperitoneal injection of the drug. The situation was entirely different when cattle were given Prothidium subcutaneously, the route of injection which is used in the field. The drug formed a depôt at the site of injection and was readily detectable near the site of injection for at least 3 months after treatment. It seems likely therefore that the prolonged prophylactic action of Prothidium is a result of the formation of a local depôt and to the powerful binding of the drug by liver tissue. This would explain the failure to find significant concentrations of Prothidium in the blood of animals which are resistant to trypanosome infection.

Williamson and Desowitz (1956) have shown that a mixture of Prothidium and suramin is less toxic to cattle and shows a greater prophylactic action than either compound alone. When Prothidium and suramin are mixed in the proportions 1:0.77 an insoluble complex is formed and it is probable that the effectiveness of the mixture is due to the formation of a better local depôt at the injection site by such an insoluble salt. Several similar complexes were prepared by these workers by mixing either quinapyramine sulphate, homidium bromide, berenil or RD 2902 with suramin. Extensive field trials were carried out on these substances by Desowitz (1957), who found that the homidium bromide-suramin complex was the most efficient prophylactic in cattle. However, he found that this substance was still highly toxic, whereas no severe local reactions or obvious toxic symptoms were found in the few animals treated with the Prothidium-suramin complex. Since the 3 cows used in the present work also showed little sign of toxic symptoms after treatment with Prothidium, it may be that the Prothidium-suramin complex could be used more safely in the field than the complex of homidium bromide and suramin. No direct comparisons have so far been made.

I would like to thank Dr. F. Hawking for suggesting this problem, and I am deeply grateful to Dr. T. S. Work for his unfailing advice and encouragement in these experiments. I am grateful to Dr. P. Walker, Dr. J. Hitchcock, and Mrs. V. Mijoric for their advice with some of the experiments. I am also much indebted to Miss B. C. Staehelin for her willing and skilful technical assistance.

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THE ANTIMICROBIAL PROPERTIES OF SOME α-AMINO-OXY-ACIDS, α-AMINO-OXY-HYDRAZIDES, ALKOXYAMINES, ALKOXYDIGUANIDES AND THEIR DERIVATIVES

BY

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A series of compounds containing an amino-oxy-group has been examined for antibacterial and antifungal activity. The amino-oxy-acids and the alkoxyamines showed little activity, but the hydrazides, notably the amino-oxydodecyl and the amino-oxytetradecyl compounds, had appreciable activity against *Staphylococcus aureus*. The alkoxydiguanides showed considerable bactericidal activity *in vitro* against Gram-positive and Gram-negative organisms and some activity against *Mycobacterium tuberculosis*. The most active compounds were those with a chain containing 10 to 14 carbon atoms, but the activity was considerably reduced in the presence of serum. They were also active against the few fungi tested. The most active compound, decyloxydiguanide, was moderately toxic when administered intraperitoneally to mice and no therapeutic activity could be demonstrated against an intraperitoneal injection of *Streptococcus pyogenes* administered 15 min. previously.

Canavanine, the *N*-amidino-derivative of an *O*-substituted hydroxylamine, is a powerful inhibitor of certain strains of *Neurospora* (Horowitz and Srb, 1948). Hydroxylamine itself shows slight antibacterial properties (Gray and Lambert, 1948), while certain alkoxyamines (I) and *N*-alkoxyguanidines (II) are active particularly against Gram-positive bacteria (Fuller and King, 1947).

$RONH_2$ (I) $\quad RONH.C(:NH).NH_2$ (II) $\quad RR'C(ONH_2).CO_2H$ (III)

$RONH.C(:NH).NH.C(:NH).NH_2$ (IV)

$RCH(ONH_2).CO.NH.NH_2$ (V)

$RCH(CO_2H).O.NH.C(:NH).NH.C(:NH).NH_2$ (VI)

The preparation of some compounds of types (I) and (III to VI) as potential antibacterials based on hydroxylamine has been described by McHale, Green, and Mamalis (1960) and by Mamalis, Green, and McHale (1960); the antibacterial properties of these compounds are now described. In order to facilitate comparisons with existing data, some of the alkoxyamines (I) of Fuller and King were re-examined. All compounds were tested *in vitro* against representative Gram-positive and Gram-negative organisms; the more active compounds were also screened against a wider range of bacteria including *Mycobacterium tuberculosis* and against some fungi. One compound was examined for its ability to protect mice against *Streptococcus pyogenes* infection.

METHODS

Antibacterial Studies in vitro.—All the compounds were screened against *Staphylococcus aureus* NCTC 4163 and *Escherichia coli* NCTC 8196 using a simple serial dilution test in nutrient broth containing Lab. Lemco 1%, Peptone (Evans) 1%, NaCl 0.5%, at *p*H 7.5. The compounds, in 0.12% aqueous solution adjusted to *p*H 6.8 to 7.0, were added in 5 ml. volumes to 5 ml. of double-strength nutrient broth to give 600 p.p.m. of the substance under test. Twofold dilutions were then made in single-strength broth. The tubes were autoclaved for 10 min. at 10 pounds/square inch pressure and, after cooling, inoculated with 0.02 ml. of a 6 hr. log-phase culture diluted 1 in 10^7. All tubes were incubated at 37° and the minimum inhibitory concentrations (M.I.C.) were observed after 48 hr. Sulphathiazole was included as a reference compound in all tests. Screening against *Streptococcus pyogenes* NCTC 8322, *Corynebacterium diphtheriae* NCTC 3989, *Eberthella typhosa* NCTC 160, *Klebsiella pneumoniae* PCI/602, and *Pseudomonas aeruginosa* NCTC 8203 was similarly carried out both in the absence and presence of 10% normal horse serum.

To determine whether the compounds were bactericidal or merely bacteriostatic, subcultures were

made from the tubes after 18 hr. incubation into large volumes of nutrient broth or on to agar plates. The bactericidal concentration was established as the minimal concentration from which living organisms could not be recovered.

For determination of activity against *Mycobacterium tuberculosis* H 37 Rv, twofold serial dilutions of the compounds in 3 ml. of Dubos and Davis Tween Albumin medium were infected with 0.02 ml. of a 10 day Dubos and Davis culture diluted 1 in 10 and incubated at 37° for 14 days. Isoniazid and *p*-aminosalicylic acid were included as standards for each batch of tests.

Antifungal Studies in vitro.—Antifungal activity was assessed using techniques essentially similar to those of Collier, Potter and Taylor (1955). Dequalinium chloride and chlorhexidine diacetate were used as reference compounds.

Antibacterial Studies in vivo.—Attempts were made to demonstrate systemic activity in mice by protection experiments using *Streptococcus pyogenes* (Richards). A culture of sufficient virulence was obtained after repeated passage through mice, and a 6 hr. log-phase culture used. An infecting dose of 0.2 ml. of a 1 in 10^6 dilution of such a culture (equivalent to 20 lethal doses) was administered intraperitoneally 15 min. before administration of the compound.

Results

Alkoxyamines, α-Amino-oxy-acids, and α-Amino-oxy-hydrazides

In confirmation of the observations of Fuller and King, we have found that the hydrochlorides of alkoxyamines (I) have only feeble antibacterial activity *in vitro* with M.I.C.s of the order 300 to 600 p.p.m. for both *Staph. aureus* and *E. coli* (sulphathiazole under the same conditions has an *in vitro* M.I.C. of 5 to 10 p.p.m.). Slightly greater activity was observed against *M. tuberculosis* (75 to 600 p.p.m.) as compared with the control compounds (0.125 to 0.5 p.p.m.). The following alkoxyamines (I) were examined: R = hydrogen (hydroxylamine), ethyl, n-propyl, isopropyl, n-butyl, n-pentyl, n-hexyl, heptyl, decyl, dodecyl, and benzyl.

The corresponding α-amino-oxy-acids (III) were slightly more active than the alkoxyamines (I) against *Staph. aureus* and *E. coli* (ca. 150 to 600 p.p.m.) while activity against *M. tuberculosis* (ca. 37.5 to 150 p.p.m.) was also somewhat greater. The following α-amino-oxy-acids (III) were examined: R = hydrogen, R′ = hydrogen, methyl, ethyl, n-propyl, isopropyl, n-butyl, n-pentyl, n-hexyl, heptyl, octyl, decyl, dodecyl, 4-cyclohexyl-butyl; R = R′ = methyl.

The possibility existed that the amino-oxy-acids might be amino-acid antagonists and this was investigated with α-amino-oxyisovaleric acid using *Leuconostoc mesenteroides* P. 60, an organism exacting towards valine, the analogous amino-acid. The growth-promoting effect of valine on a valine-deficient synthetic medium was found to be stimulated rather than inhibited by the amino-oxy-analogue, which in fact exerted a slight but significant sparing effect on valine utilization. In the absence of valine, the amino-oxy-compound was itself utilized to a limited extent, about 1,000 μg. being needed to replace 20 μg. valine.

Some α-amino-oxy-hydrazides (V; R = n-hexyl, heptyl, octyl, decyl, and dodecyl) were tested for antitubercular activity *in vitro* and shown to be rather more active (M.I.C.s 9 to 75 p.p.m.) than the analogous acids. All but two of the hydrazides showed little activity against *Staph. aureus* and *E. coli* (about 300 p.p.m.), the exceptions being (V; R = dodecyl and tetradecyl) which inhibited growth of *Staph. aureus* at 30 and 37.5 p.p.m. respectively.

Alkoxy- and Arylmethoxy-diguanides in vitro

While the alkoxyamines (I), the acids (III), and the hydrazides (V) showed little or no increase in activity against *Staph. aureus* and *E. coli* with increasing molecular weight, the same was not true of the alkoxydiguanides (IV). The hydrochlorides of a series of diguanides (IV; R = hydrogen, ethyl, n-butyl, isobutyl, n-pentyl, n-hexyl, heptyl, octyl, nonyl, decyl, undecyl, 2-methyldecyl, dodecyl, tetradecyl, and hexadecyl) were examined. The M.I.C.s for both the above two organisms were found to increase rapidly with increasing alkyl chain length, reaching a maximum (0.6 to 1.25 p.p.m.) at C_{10} to C_{12}. The highest members of the series showed reduced activity, perhaps associated with decreased aqueous solubility. The activity of these compounds against *M. tuberculosis* followed a similar pattern with a maximum effect at C_{10} to C_{12} (5 p.p.m.). Branching of the alkyl chain did not affect activity.

Introduction of a carboxyl group into the diguanides to give (VI; R = n-pentyl, n-hexyl, heptyl, octyl, and decyl) resulted in complete loss of *in vitro* activity.

Hydrochlorides of arylmethoxydiguanides were found, in general, to be less active than the alkoxydiguanides. Of the following which were tested [IV; R = benzyl, *p*-chloro-, *p*-bromo-, and 3,4-dichloro-benzyl, 6-chloro-1,3-benzodioxan-8-yl-methyl, 1- and 2-naphthylmethyl, and 1-bromo-2-naphthylmethyl], 2-naphthyl-methoxydiguanide hydrochloride was the most active against *Staph. aureus* (25 p.p.m.) and *E. coli* (12.5 p.p.m.) [cf. decyloxydiguanide dihydrochloride (1.25 p.p.m.) for both organisms].

TABLE I

MINIMAL EFFECTIVE CONCENTRATIONS (P.P.M.) OF SOME ACTIVE ALKOXYDIGUANIDES IN THE PRESENCE AND ABSENCE OF 10% SERUM

Column a: in broth. Column b: in broth + 10% normal horse serum.

	Alkoxydiguanides RONH.C(:NH).NH.C(:NH).NH_2								Reference Compounds					
	$R=C_8H_{17}$ No. 129		$R=C_{10}H_{21}$ No. 130		$R=C_{12}H_{25}$ No. 133		$R=C_{14}H_{29}$ No. 134		Sulphathiazole		Dequalinium Chloride	Chlorhexidine Diacetate
	a	b	a	b	a	b	a	b	a	b	a	a
Staph. aureus 4163	10	50	0·6	37·5	1·5	75	3	75	5	600		
Strept. pyogenes 8322	2·5	12·5	1·25	12·5	2·5	12·5	10	37·5	>300	>1,200		
C. diphtheriae 3989	2·5	12·5	1·25	12·5	0·6	12·5	0·6	12·5	5	6·25		
E. coli 8196	10	37·5	1·25	37·5	1·5	75	6·25	150	10	>600		
Kleb. pneumoniae 1/602 ..	12·5	40	5	30	5	40	100	150	300	400		
Sal. typhii 160	12·5	30	2·5	40	2·5	50	5	>50	25	40		
Ps. aeruginosa 8203	50	150	12·5	300	>200	>600	>200	>600	>600	>1,200		
Microsporon canis	20		2·5		2·5		20				<0·6	0·6
Candida albicans	>20		10		20		>20				<0·6	10
Trichophyton mentagrophytes ..	20		5		10		>20				<0·6	5
Epidermophyton floccosum ..	10		2·5		2·5		20				<0·6	5

The table shows the activity of the four most active alkoxydiguanides against a wider range of organisms, the least susceptible being *Ps. aeruginosa*. In the presence of 10% serum, the activities were diminished sharply, the M.I.C.s being increased from 5 to 50 fold. With each of the bacteria listed in the table, it was confirmed that the inhibition was bactericidal and not merely bacteriostatic.

The table also shows that antifungal and antibacterial activities run parallel, the peak effect being reached with the C_{10} and C_{12} compounds.

Toxicities

No adverse effects were noted with any of the four compounds in the table when neutralized suspensions (in 0.5 ml.) were injected subcutaneously into mice (19 to 21 g.) in doses up to 1,000 mg./kg. The mice were killed after 9 days and examined: it was found that the C_{10}, C_{12} and C_{14} compounds remained undissolved in the subcutaneous tissue. The more soluble acetate of decyloxyguanide (C_{10}) when injected subcutaneously at 1,000 mg./kg. produced no systemic effects, but severe necrosis developed at the site of the injection. The intraperitoneal toxicity of the latter compound was determined in mice; the LD50 (Miller and Tainter, 1944) was found to be 20 ± 2.5 mg./kg.

In vivo *Experiments with Diguanides*

The activity *in vivo* of decyloxydiguanide acetate (IV; R=decyl), the most active member of the series, was examined in groups of mice infected with *Strept. pyogenes* (Richards). Fifteen min. after intraperitoneal infection with 0.2 ml. of a 6 hr. log-phase culture, the compound was administered intraperitoneally at 10 mg./kg. The treated and control mice died within three days and no evidence for any prolongation of survival times was observed. Although no attempt has been made to isolate metabolic products, it would appear, therefore, that the compound

is excreted or metabolized too rapidly to show any protective effect; diguanides are known to react rapidly with ketones and keto-esters to form cyclic products.

We are indebted to Dr. J. H. Humphrey for the culture of *Streptococcus pyogenes* (Richards), to Dr. H. O. J. Collier and Dr. F. L. Rose for the samples of dequalinium, chloride and chlorhexidine diacetate respectively, and to Mr. K. J. Stevens for valuable technical assistance.

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ANALGESIC ACTION OF ETHYL 4-PHENYLPIPERIDINE-4-CARBOXYLATES WITH OXYGENATED 1-SUBSTITUENTS

BY

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WITH AN ADDENDUM BY R. E. LISTER AND R. P. STEPHENSON

(RECEIVED DECEMBER 17, 1959)

The analgesic potency of a series of new compounds related to pethidine has been measured in rats. The replacement of the methyl group on the nitrogen atom of pethidine by groups containing ether linkages produces an increase in potency. Two different positions for the oxygen atom are particularly beneficial, but the effects, in these two positions, appear to be exerted in different ways and are not additive. Simple toxicity tests in mice and rats and tests on respiration in rats of five of the more potent compounds revealed no unexpected adverse action.

Until about 1955 it was accepted (see Braenden, Eddy, and Halbach, 1955) that in analgesics of the pethidine type, that is, in 4-phenylpiperidines with an alkoxycarbonyl, acyl, or acyloxy group in the 4-position, the most desirable substituent on the nitrogen atom was a methyl group. More recently, it has been shown that more active compounds may be prepared by introducing other substituents on the nitrogen atom (Millar and Stephenson, 1956; Perrine and Eddy, 1956; Weijlard *et al.*, 1956; Elpern, Gardner, and Grumbach, 1957; Janssen *et al.*, 1959).

The present study is concerned with derivatives of pethidine in which the *N*-methyl group is replaced by substituents carrying at least one ether or thioether function. The substances tested were synthesized by Frearson and Stern (1958) and Frearson, Hardy, and Stern (1960), and are listed in Tables I to IV.

METHODS

Analgesia (Green and Young, 1951).—The pressure on the tail needed to make a rat squeak was measured: drug was injected subcutaneously in the flank in 0.2 ml. saline per 50 g. body weight, and the measurement repeated 6 times at 10 min. intervals. Analgesia was assessed in terms of the index of analgesia (Millar and Stephenson, 1956), which was obtained by dividing the pre-injection pressure by each post-injection pressure. The mean of the 6 indices gave a measure of the effect in that rat. The highest pressure applied to the tail was 3 times the average control value; if this did not produce a squeak the index was taken as zero.

The new compounds were first tested, at several dose levels, in groups of 3 rats. From the results of these tests, 2 doses, expected to be equipotent with 2 and 4 mg./kg. of morpheridine (TA 1), were selected. These 4 doses were each administered to 4 or 5 rats. This test was repeated, so that each dose was given to about 9 animals. Most of the results listed in Tables I to IV were obtained from these simple tests.

To obtain a more accurate measure of the potency of some of the more active compounds and of the relations within several small series of close analogues, four-way cross-over tests were performed. High and low doses of 4 drugs were used in each trial. Each of 24 rats received each drug, 2 at the high and 2 at the low dose level. Each dose was thus administered to 12 rats and the design confounded the variance due to differences in slope and the variance due to differences between rats. Since there were 8 doses, the rats were housed 8 to a cage, and on each day of the test each dose was given to 1 rat from each of the 3 cages. Three randomized versions of the design given by Finney (1952) were used to determine the order of treatment.

In Tables I to III the results from the cross-over tests are indicated by the inclusion of fiducial limits ($P=0.95$).

Respiratory Depression.—Rats were anaesthetized with urethane. Respiration was recorded by a modification of Gaddum's method (1941) using a Krogh volume recorder converted into a sensitive pressure recorder (Paton, 1949). Drugs were injected into the femoral vein.

Peristaltic Reflex.—Trendelenburg preparations of the guinea-pig ileum were set up as described by Schaumann (1955). Fresh tissue and also pieces

stored for 24 hr. at low temperature were used. The substances were added to the bath (40 ml.) 1 min. before the intraluminal pressure was raised. Three doses of each drug were given so that the contractions were reduced by between 20 and 50%.

Toxicity Tests.—In acute experiments, the drugs in saline, 4 ml./kg., were injected intravenously in mice. Each dose was injected into 5 mice, and if possible the LD50 for each drug was determined. Some of the hydrobromides could only be dissolved in warm saline and the LD50s given for these substances may not be very accurate. (Attempts to use ethanol/water mixtures were abandoned when it was found that morpheridine, injected in ethanolic solution, showed increased toxicity.)

In subacute experiments, 2 groups of 10 newly weaned rats were weighed and given subcutaneous injections daily over 12 days. One group, kept as controls, received saline 4 ml./kg. The other group received 5 times the larger dose used in the analgesic cross-over tests. To obtain some indication of the development of tolerance, an analgesic test was performed on the thirteenth day; suitable doses of the drug under test were given to equal numbers of control and treated animals.

The animals were then killed and blood smears taken. Samples of liver, kidney, spleen, heart, lung, brain, and bone marrow were removed and fixed for pathological examination. Some rats died during these tests and were usually eaten by the survivors, so that they were not available for examination.

Results

Analgesia.—Since the compounds in the present series are similar to morpheridine (TA 1) (Millar and Stephenson, 1956) and since, in the test used, pethidine and morpheridine do not give parallel dose-effect curves, we have compared the new substances with morpheridine rather than pethidine.

A number of compounds in which the *N*-substituent was of the type $-[CH_2]_2.OR$ were tested. The results on those in which R was aliphatic, together with other aliphatic substituents of the type $-[CH_2]_n.OC_2H_5$, are listed in Table I. In the first category, the most active compound was the ethoxyethylnorpethidine ($R=C_2H_5$; TA 24), which was as active as morpheridine. This compound is also one of the more active of the series $-[CH_2]_n.OC_2H_5$. The most active compound, up to and including $n=6$, is TA 33 where $n=4$. Independent observations by Morren and Strubbe (1957) agree with our results for TA 24, TA 25 and TA 36.

In Table II are listed compounds in which the *N*-substituent is again $-[CH_2]_n.OR$, but where R now contains an aromatic residue. Both the phenoxy- (TA 27) and the benzyloxy-ethylnorpethidine (benzethidine; TA 28) were rather more potent than morpheridine, but the 2-naphthyloxynorpethidine (TA 54) was much less potent. A number of substituted phenoxy-compounds were tested; all were less potent than the parent compound, TA 27. The phenoxybutylnorpethidine (TA 62) was about one-third as potent as TA 27.

Table III shows the results obtained with a miscellaneous group of compounds in which the substituent on the nitrogen atom contains more than one ether grouping and/or a cyclic ether function. The most active member of the present series, furethidine (TA 48), has both of these properties (but see addendum).

In Table IV are collected the results with the sulphur analogues of TA 24, TA 27, and TA 29,

TABLE I

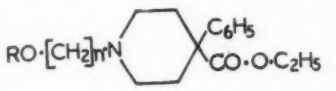
ANALGESIC ACTIVITY OF ALKOXYALKYLNORPETHIDINES

The bases, dissolved in 0.1 N HCl, were examined, except TA 25 and TA 42 which were available as the hydrobromides. Analgesic potency is compared with morpheridine (TA 1) (=100). In Tables I to III, the results based on the 6 cross-over tests may be identified by the inclusion in parentheses of fiducial limits (P=0.95). The superscript numerals identify the number of the cross-over test so that the more immediately comparable figures may be identified. The 3rd and 5th tests did not include morpheridine, since the purpose was to compare small homologous series, and the fiducial limits, though quoted for ease of comparison in terms of the potency of morpheridine, are strictly valid only for comparison within the group of compounds in the particular cross-over test.

TA No.	*n*	R	Relative Analgesic Potency (Limits) in Rats	Intravenous LD50 (Mice) (mg./kg.)
29	2	Methyl	35 (28, 43)[5]	20
24	2	Ethyl	103 (85, 125)[1,3,5]	20
34	2	Propyl	44 (36, 54)[5]	22
35	2	Isopropyl	50	12
36	2	Butyl	42 (35, 50)[5]	15
42	2	Cyclohexyl	25	12
25	3	Ethyl	38 (31, 46)[3]	15
101	3	Methyl	12	—
33	4	Ethyl	265 (191, 368)[3]	15
32	5	,,	73 (56, 94)[3]	17
69	6	,,	47 (38, 61)[6]	—

TABLE II

ANALGESIC ACTIVITY OF ARYLOXYALKYLNORPETHIDINES



The hydrobromides of the bases (dissolved in saline) were examined. An asterisk (*) indicates that the hydrobromide was not sufficiently soluble so the LD50 may be inaccurate.

TA No.	*n*	R	Relative Analgesic Potency (Morpheridine=100) in Rats	Intravenous LD50 (Mice) (mg./kg.)
27	2	Phenyl ..	140 (115, 169)[1]	15
62	4	Phenyl ..	40	17
37	2	*m*-Chlorophenyl	40	30
30	2	*p*-Chlorophenyl ..	15	20
60	2	*o*-Methoxyphenyl ..	10	30
39	2	*m*-Methoxyphenyl ..	40	15
41	2	*p*-Methoxyphenyl	20	25
45	2	*o*-Tolyl ..	11 (7, 18)[4]	20*
44	2	*m*-Tolyl	31 (22, 43)[4]	12*
47	2	*p*-Tolyl ..	17 (13, 24)[4]	15
55	2	*o*-Nitrophenyl	5	—
53	2	*m*-Nitrophenyl	7	50
46	2	*p*-Nitrophenyl	10	25*
51	2	4-Diphenylyl	15	40
40	2	*p*-Acetamidophenyl ..	30	15
43	2	*p*-Ethoxycarbonylphenyl	5	40
54	2	2-Naphthyl	5	—
28	2	Benzyl (benzethidine) ..	145 (117, 177)[1]	7·5

all of which were less active than the corresponding oxygen ethers.

In view of the activity of furethidine and of morpheridine, the "model" substance 2-morpholinoethyl tetrahydrofurfuryl ether was examined: it had no analgesic potency.

In one of the cross-over tests the analysis of variance showed deviation from parallelism significantly greater than chance expectation. This was caused by TA 49, which produced much less increase in effect with dose than the other compounds. In other tests TA 33 and TA 45 also appeared to have lower slopes than the rest of the compounds, but the difference was not significant.

None of the compounds in this series has hitherto been compared directly with morphine and, although many comparisons of morphine and pethidine in rats have been published, we thought it desirable to compare morpheridine and morphine in our conditions. Morpheridine and its analogues usually showed definite peak activity 10 to 20 min. after injection whereas morphine maintained its action longer, so that the result of any comparison depended on its duration. Our test of 1 hr. duration showed that morpheridine was 1.2 times as potent as morphine and furethidine was about 7 times as potent as morphine.

Respiration.—The substances examined all depressed respiratory minute volume when given intravenously in rats anaesthetized with urethane. The rapid onset and short duration of the depression was very similar to that produced by pethidine, but differed markedly from that caused by morphine which was rather slow in onset and prolonged. The potent analgesics morpheridine, benzethidine, furethidine, TA 27, and TA 33 produced respiratory depression in smaller doses than pethidine, but the difference was usually less than the difference in analgesic potency. Indeed, furethidine appeared to have an appreciable advantage over pethidine, though this may reflect the different conditions and route of administration rather than any separation of analgesic action from respiratory depression.

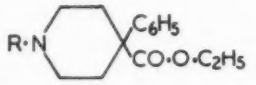
Peristaltic Reflex.—The derivatives depressed both the emptying and the preparatory phases of the peristaltic reflex and the relative activity of some of the compounds was assessed on the preparatory phase. The results, listed in Table V, correspond with those of analgesic tests. Results on fresh tissues differed slightly from those on stored preparations, possibly because of the occurrence of the emptying phase in the fresh tissue.

Toxicity Tests.—The acute intravenous LD50 of most of the substances is listed in Tables I to IV. Death was apparently due to respiratory depression and the convulsions which occur with pethidine were not observed with these derivatives.

In order to obtain some indications of any pathological lesions produced by the more potent substances, doses 5 times as great as the high dose of the analgesic cross-over test were given daily for 12 days. With each of the 6 compounds tested this dose was sufficient to kill at least one of the 12 rats. These deaths were presumed to be

TABLE III

ANALGESIC ACTIVITY OF NORPETHIDINE DERIVATIVES CONTAINING A CYCLIC ETHER GROUPING OR MORE THAN ONE ETHER GROUPING



All substances except TA 61 were available as bases and were dissolved in dil. HCl. TA 61 was available as the hydrobromide and was dissolved in saline.

TA No.	R	Relative Analgesic Potency (Morpheridine=100) in Rats	LD50 (Mice) (mg./kg.)
57	Tetrahydrofurfuryl	15	45
20	2-(Tetrahydropyran-4-yl)ethyl ..	90	30–40
48	2-(Tetrahydrofurfuryloxy)ethyl (furethidine) ..	729 (564, 960)[2]	25
70	3-(Tetrahydrofurfuryloxy)propyl ..	35	—
63	4-(Tetrahydrofurfuryloxy)butyl ..	125	7·5
49	2-(Tetrahydropyran-2-ylmethoxy)ethyl	265 (167, 420)[2]	10
65	2-(Tetrahydropyran-2-yloxy)ethyl ..	17 (13, 22)[6]	—
64	4-(Tetrahydropyran-2-yloxy)butyl ..	35 (27, 45)[6]	—
38	2-(2-Ethoxyethoxy)-ethyl	39 (30, 50)[2]	20
61	2-(2-Phenoxyethoxy)-ethyl	30	20

TABLE IV

ANALGESIC ACTIVITY OF NORPETHIDINE DERIVATIVES CONTAINING A THIOETHER FUNCTION

TA No.	R	Relative Analgesic Potency (Morpheridine =100) in Rats	LD50 (Mice) (mg./kg.)
59	Methyl	15	40
58	Ethyl	5	50
56	Phenyl	10	—

due to respiratory depression. The survivors did not show any marked retardation in growth compared with simultaneous controls. The histological examination revealed no evidence that the drugs were harmful. Occasional tissues showed a variety of abnormalities, but these occurred as frequently in the controls as in the treated animals. No signs of kidney, liver, or bone marrow damage were observed.

The analgesic tests on the rats after the 12-day treatment showed signs of some—but usually not very much—tolerance. In Table VI the tolerance may be judged from the difference beween the analgesic effects produced in the treated rats and those in the controls tested simultaneously. The number of animals tested was too small to permit firm conclusions, but TA 24 seemed to produce less tolerance than the other drugs.

DISCUSSION

The substances tested by Millar and Stephenson (1956) were analogues of pethidine in which the *N*-methyl group was replaced by tertiary aminoalkyl groups, most of which were in a ring. The only compounds showing activity were those in which the ring also contained an ether linkage. Our present findings show that neither the additional nitrogen atom (for example, TA 20) nor the ring structure (for example, TA 24, TA 33) are necessary and that enhanced activity may be achieved by inclusion of simple ether substituents at the 1-position.

It is not easy to see why such an ether grouping should have this effect. It is chemically inert and is some distance from what is normally considered to be the active part of the molecule. Two possibilities are that the oxygen atom provides a new point of attachment between the molecule and its site of action, or that it modifies the properties of the molecule as a whole and, for example, facilitates access to the site of action. The latter possibility does not appear likely because: (1) The *p*K values of the bases are all about 7.5, irrespective of potency. (2) One additional carbon atom, while not expected to affect the physical properties greatly, changes the analgesic potency very considerably. (3) Isomers may differ widely in potency (for example, TA 36 and 33, TA 24 and 101, and furethidine and TA 65). Clearly, the efficiency of the oxygen atom in enhancing potency depends rather critically on its position.

In the alkoxyalkyl series (Table I) where only the oxygen atom is involved, two compounds (TA 24 and TA 33) are more active than the rest. In these, the oxygen atom is the third or fifth

TABLE V

RELATIVE DEPRESSANT POTENCY ON PREPARATORY PHASE OF PERISTALTIC REFLEX (MORPHERIDINE=1)

Substance	Fresh Intestine	Stored Intestine
TA 25	0·4	0·5
TA 27	1·0	1·3
Benzethidine (TA 28)	1·0	1·4
TA 33	2·6	3·1
Furethidine (TA 48)	6·5	7·8
Pethidine	0·3	0·7
Morphine	1·9	5·0

atom from the nitrogen atom. TA 33, where the oxygen atom is at much the same distance from the nitrogen atom as in morpheridine and TA 20, is more potent than TA 24.

In the phenoxyalkyl series (Table II), on the other hand, TA 27, in which the oxygen atom is the third atom, is more potent than TA 62, in which it is the fifth atom from the nitrogen atom. The proximity of the phenyl group provided an opportunity to study the effect of changes in the electronic environment of the oxygen atom in TA 27. However, the potency was reduced, whatever the inductive or mesomeric effects of the substituents which were introduced into the phenyl group. This general reduction of potency in the phenoxyethylnorpethidines contrasts with the variation observed by Elpern *et al.* (1957) in the substituted phenylethylnorpethidines. In fact, the presence of the oxygen atom in the phenoxyalkyl series appears to be unnecessary: 3-phenylpropylnorpethidine (Elpern *et al.*, 1957), in which the oxygen atom of TA 27 is replaced by a methylene group, is more potent than TA 27. It thus appears that the phenyl group is more important than the oxygen atom in this series. This may account for the fact that the peak activity in the phenoxyalkylnorpethidine series occurs when the oxygen atom is the fourth atom from the nitrogen atom (Sterling Drug Inc., 1959), in contrast to our finding in the alkyloxyalkyl series.

The most potent of the present compounds is furethidine, which in addition to an open-chain ether linkage has a tetrahydrofuran ring. This cyclic ether grouping is obviously very efficient in enhancing potency; the potency of the corresponding tetrahydropyran (TA 49) is lower and that of the open-chain analogue (TA 38) is lower still. Mr. P. M. Frearson has pointed out to us that this order corresponds to that of the electron

TABLE VI

SUBACUTE TOXICITY AND DEVELOPMENT OF TOLERANCE

* The lower the index the higher the analgesia.

Substance	Daily Dose/ kg.	Deaths	% Increase in Wt. of Survivors	Analgesic Tests after 12 Days' Treatment			
				Low Dose (mg./kg.)	Mean Analgesic Index*	High Dose (mg./kg.)	Mean Analgesic Index*
Saline	4 ml.	0	62·6	1·5	0·72	3	0·43
TA 27	15 mg.	1	54·2	1·5	0·86	3	0·56
Saline	4 ml.	0	65·7	2	0·75	4	0·39
TA 24	20 mg.	3	68·6	2	0·81	4	0·43
Saline	4 ml.	0	94·8	0·25	0·66	0·5	0·34
Furethidine (TA 48) ..	2·5 mg.	1	84·1	0·25	0·85	0·5	0·59
Saline	4 ml.	0	82·6	2	0·80	4	0·44
TA 20	20 mg.	2	66·9	2	0·94	4	0·79
Saline	4 ml.	0	34·4	1·5	0·77	3	0·39
Benzethidine (TA 28) ..	15 mg.	5	45·6	1·5	0·98	3	0·72
Saline	4 ml.	0	37·3	0·5	0·83	1	0·61
TA 33	5 mg.	2	59·5	0·5	0·96	1	0·73

donor properties (Searles and Tamres, 1951), and it may well be that an oxygen atom in this position donates electrons at the site of action, for example, by hydrogen-bond formation.

An oxygen atom nearer to the nitrogen atom, however, does not produce a greater effect if it is part of a ring: TA 57 is much less active than TA 24. Indeed, the relatively high potency of TA 24 seems to be somewhat anomalous; it stands out of the otherwise regular rise and fall of potency as the alkyl group is lengthened in the ethoxyalkylnorpethidines, and this is not just a matter of overall chain length since TA 101 is much less potent than TA 24. Furthermore, when some group conferring increased potency is present at a greater distance, an oxygen atom at the third atom from the nitrogen atom appears to reduce potency. This occurs in the phenoxyalkyl series (see above) and in the case of TA 38 and TA 32. The most active compound we have tested, furethidine, has such an oxygen atom. Dr. Stern and his colleagues have now prepared analogues without this oxygen atom, and these have been tested by Dr. Lister. His results—given in an addendum to this paper—show that the presence of the ether linkage nearer the nitrogen atom slightly reduced the advantage conferred by the more distant tetrahydrofurfuryl group.

The nature and shape of possible receptors for morphine-like analgesics have been suggested by Beckett and Casy (1954) and by Barlow (1955). These must be considered as part of an extended surface. Our findings could well be explained by the presence on this extended surface of an area able to accept electrons, situated at a distance about equal to the length of a 5-carbon chain from the point at which the nitrogen atom is supposed to be accommodated.

Such an additional point of attachment would be expected to provide additional stability or rigidity to the complex formed between drug and receptor. Increased electron density at approximately the right distance from the nitrogen atom appears to be a common feature of all the norpethidine derivatives recently shown to exceed pethidine in potency. These include the phenylpropyl-, 4-pyridylethyl-, cinnamyl-, *p*-aminophenylethyl- (Elpern *et al.*, 1957), phenoxypropyl- (Sterling Drug Inc., 1959), 3-phenyl-3-oxopropylnorpethidine (Janssen *et al.*, 1959), as well as morpheridine, benzethidine, TA 20, TA 33, furethidine, TA 98 and TA 103.

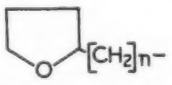
Many of the compounds we have tested are appreciably more potent than pethidine and the question arises whether any one of them might be better than pethidine in clinical use. The capacity to produce undesirable side-effects in analgesics usually runs roughly parallel with the capacity to produce analgesia. High potency in itself is therefore no great virtue (though pethidine is so weak that doses of 100 mg. are often administered and 2 ml. of solution is required to avoid tissue damage). The side-effects can be finally assessed only in man, and even if one compound could be shown to be superior to the others in animal tests, it does not follow that it would also be better in man. We have not attempted to decide which is best in animals and have contented ourselves with showing that five of the compounds did not depress respiration unduly, and did not produce pathological changes in the subacute toxicity tests, in the hope that these five may all be tested in man. Such a test has, in fact, been undertaken (Cahal, D. A., personal communication).

We wish to thank Dr. A. E. Stuart, Department of Pathology, Edinburgh University, for the pathological examinations. We also thank Dr. E. S. Stern and J. F. Macfarlan and Co. Ltd., who supplied the compounds, more particularly since they synthesized a number of compounds which were likely to be of theoretical rather than commercial interest. J. F. Macfarlan and Co. also provided a postgraduate grant which enabled A. M. J. N. B. to do this work.

ADDENDUM

By R. E. Lister and R. P. Stephenson

Since the completion of the work described in the main body of the paper a further short series of compounds has been prepared and assessed for analgesic activity. The *N* substituent of these compounds is



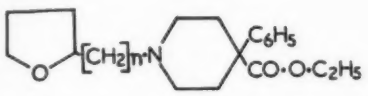
n being 1 to 5 inclusive.

Analgesic activity was determined by a method differing slightly from that described above, namely, by the quantal method of Green and Young (1951), and ED50 for analgesia being determined. Both the method of Millar and Stephenson (1956) and the method for the present series use the same pain stimulus and the same animal, namely, pressure on the tail of young rats, but somewhat different results were obtained in the comparison of morpheridine and furethidine.

All five new compounds possessed marked analgesic activity (Table VII); the activity increased with increasing chain length up to a maximum at $n=3$ and 4, but further increase in n to 5 reduced the activity. The derivatives

TABLE VII

ANALGESIC ACTIVITY AND TOXICITY OF 1-(ω-TETRAHYDROFURFURYLALKYL)NOR-PETHIDINES



Substance	*n*	Sub-cutaneous ED50 in Rats (mg./kg.)	Relative Potency (Morpheridine =100)	Intra-venous LD50 in Mice (mg./kg.)
TA 57 ..	1	26	8	45
TA 109 ..	2	3·7	55	23
TA 98 ..	3	0·3	670	11
TA 103 ..	4	0·3	670	11
TA 108 ..	5	1·6	125	12
Pethidine ..	—	7·2	30	58
Morpheridine (TA 1) ..	—	2·0	100	45
Benzethidine (TA 28) ..	—	0·9	220	11
Furethidine (TA 48) ..	—	0·4	500	15

in which $n=3$ or 4 were more potent than furethidine which has an ether linkage in the alkyl side-chain. They may be formally derived from furethidine by omission of the open-chain ether linkage, and by its replacement with a methylene group. Thus in presence of the cyclic ether grouping of furethidine, the open-chain ether oxygen in the 3′-position is of no importance in producing analgesia. This finding supports the view that the most important factor enhancing the analgesic potency is an electron-donating group at a distance of about 5 to 6 carbon atoms from the nitrogen atom.

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Brit. J. Pharmacol. (1960), **15**, 254.

PHARMACOLOGICAL ACTIONS OF TWO NEW PETHIDINE ANALOGUES

BY

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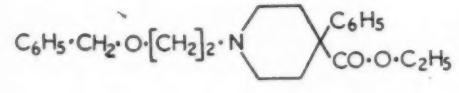
From the Research Department, J. F. Macfarlan & Co., Edinburgh, 8

(RECEIVED DECEMBER 17, 1959)

The behaviour of two new analgesics, benzethidine and furethidine, in a number of different tests has been compared with that of pethidine. Some differences in side-effects at equi-analgesic dosage were observed, particularly a reduction in histamine release.

The synthesis of a number of *N*-substituted derivatives of pethidine has recently been reported by Frearson and Stern (1958) and Frearson, Hardy and Stern (1960). Many of these compounds have been shown by Millar and Stephenson (1956) and by Blair and Stephenson (1960) to have marked analgesic activity. Two potentially useful analgesics of this series have now been compared with pethidine. These compounds are benzethidine (I; ethyl 1-(2-benzyloxyethyl)-4-phenyl-piperidine-4-carboxylate) and furethidine (II; ethyl 4-phenyl-1-(2-tetrahydrofurfuryloxyethyl)-piperidine-4-carboxylate).



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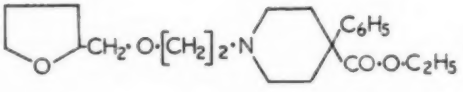

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METHODS

Analgesia.—The elevation of pain thresholds in a group of 10 weanling rats (4 to 6 weeks old) was measured 30 min. after the subcutaneous injection of the analgesic, or 1 hr. after oral administration, by a modification of the method of Green and Young (1951). The pain threshold of each rat was determined before and after administration of the drug; a squeak rather than struggle was taken as the end point. Any animal in which the pain threshold was raised to double or more than that of its initial value was judged to be showing significant analgesia. The ED50 and 95% limits of error were calculated using a standard probit analysis.

Respiration.—The respiratory depressant action on unanaesthetized rats was determined by measuring the frequency of respiration before and 30 min. after the subcutaneous injection of the drugs. The mean respiratory frequency per min. of groups of 10 young rats was determined by counting the respiratory rate of the animals very loosely restrained in a perspex tube.

The effect upon respiratory minute volume and respiratory rate was studied in anaesthetized dogs, cats and rabbits by the method of Paton (1949). The cats and dogs were lightly anaesthetized by intraperitoneal injection of pentobarbitone sodium 35 to 40 mg./kg. Rabbits were anaesthetized with urethane 1.5 g./kg. injected intravenously.

Cardiovascular System.—The direct effect of the drugs on the blood pressure of the anaesthetized rabbit, cat and dog was studied. Heart rate and electrocardiogram were recorded with a direct writing Ediswan electrocardiograph. The effect of the drugs on the normal vascular responses to noradrenaline, adrenaline, histamine, acetylcholine, nicotine and vasopressin was recorded.

Behaviour and Activity.—The effects on normal behaviour and activity of the mouse, rat, cat, dog and monkey were studied. Mice in which hyperkinesia had been induced by $\beta\beta$-iminodipropionitrile after the method of Thuillier and Burger (1954) were treated with benzethidine, furethidine and pethidine and the effect on activity was recorded semi-quantitatively using a jiggle cage.

Antitussive Action.—Cough was induced in cats lightly anaesthetized with pentobarbitone sodium (35 mg./kg.) either by electrical stimulation of the superior laryngeal nerve (Green and Ward, 1955) or by mechanical irritation by means of a polythene tube passed down the trachea as far as the carina. The cough was recorded with a spring-loaded writing lever on a kymograph by means of thread tied to the abdominal wall in the region of the xiphisternum.

Prolongation of Barbiturate-induced Sleep.—Mice were injected subcutaneously with twice the analgesic ED50 20 min. before the intraperitoneal injection of

75 mg./kg. of hexobarbitone sodium. The time from injection of the hexobarbitone to the recovery of the righting reflex was taken as the sleeping time.

Anticonvulsant Action.—Convulsions were induced in mice by the intravenous infusion of leptazol 20 min. after the subcutaneous injection of the ED50 of the analgesic.

Emetic Action.—The drugs were injected in ascending doses into the gluteal muscle of mongrel dogs 0.5 hr. after feeding. The criterion of vomiting was taken as the expulsion of stomach contents.

Mydriatic Effect.—The pupil size of mice before and after subcutaneous and local application of the drug was determined by measuring the pupil diameter with a low power microscope.

Local Anaesthetic Action.—The local anaesthetic action was determined in guinea-pigs, after the intradermal injection of the analgesics, by the method of Bülbring and Wajda (1945), and compared with procaine as a standard. Qualitative assessments in man were also made.

Histamine Release.—The histamine-releasing power was determined in man by injecting the drugs in 0.1 ml. of normal saline intradermally into the skin of the volar surface of the forearm. The area of the weal produced was measured using squared paper (Bain, 1949). Weal area was plotted against log dose.

Gastro-intestinal Effects.—The antagonistic effects of the three drugs against contractions of the guinea-pig ileum induced by acetylcholine, histamine and barium chloride were determined. The preparation described by Trendelenburg (1917) was used to study effects on the peristaltic reflex.

The influence on intestinal motility in mice was studied by comparing the effects of injection of the drugs and a control solution on the weight of faecal pellets passed by 4 groups of 8 mice. A cross-over design was used and each group was given each treatment. The faeces from each mouse were collected over a period of 2 hr. and the means for each group compared statistically.

The effect on gastro-intestinal propulsion of the three drugs under test was also examined using the method described by Green (1959). For this test young rats were used and were kept on a diet of protein hydrolysate for 2 days, being starved for 2 hr. before the commencement of the experiment.

Antidiuretic Action.—This was determined using rats which had been loaded with water by stomach tube at 5% body weight and 1 hr. later with a further 5% body weight of 12% ethanol. The bladder and jugular vein were cannulated and the urine output was measured at 4 min. intervals. All drugs were given by intravenous injection. Antidiuretic potency was calculated by the method of Dicker (1953).

Acute Toxicity.—The LD50 of the three compounds was determined after intravenous injection in mice and oral and subcutaneous injection in rats.

Subacute Toxicity.—This was determined over a period of 12 weeks on 4 groups of 20 male rats. Newly weaned rats were injected daily with furethidine, 1 mg./kg.; benzethidine, 5 mg./kg.; pethidine, 25 mg./kg.; and normal saline, 4 ml./kg. by the intraperitoneal route.

Each animal was weighed daily and any gross toxic symptoms were recorded. Blood samples were taken from randomly selected rats every 2 weeks. The samples were examined and haemoglobin level, red cell count and total white cells determined. Differential white cell counts were performed for each group at the end of the eleventh week of the test.

After 12 weeks, 10 rats from each group were killed and sections of the liver, spleen, lung, kidney, adrenal gland, bone marrow of the femur and skin at the site of injection were examined for pathological changes.

RESULTS

Analgesia.—Both benzethidine and furethidine had a considerably greater analgesic action in rats than pethidine. The activity of the three compounds is compared in Table I. All these compounds showed a similar duration of action by either route. The ratios of analgesic activities by oral and by subcutaneous administration appear to be similar, and values obtained are in satisfactory agreement with those of Blair and Stephenson (1960).

Respiration.—All three compounds produced respiratory depression in unanaesthetized rats, and in the anaesthetized rabbit, cat and dog (Table II).

In unanaesthetized rats a linear relationship between log dose in mg./kg. and the log of the difference in respiratory rates before and after the drug was obtained. The dose-response curves for the three drugs showed no significant deviation from parallelism ($P>1.0$). Similar relationships between dose and respiratory depression were obtained when the effect on the minute volume of anaesthetized cats was studied. The relative potencies of the drugs as respiratory depressants are shown in Table II.

Nalorphine and levallorphan both reversed the respiratory depression which followed the administration of pethidine, furethidine, and benzethidine.

Cardiovascular System.—All three compounds produced a fall in blood pressure when injected intravenously into the anaesthetized rat, cat and dog. A qualitative difference was observed, however, when the drugs were given in the ratio of their analgesic doses. Pethidine produced an initial short-lasting rapid fall of blood pressure which was followed by a secondary more prolonged fall. With benzethidine and furethidine this secondary fall in blood pressure was much less, or frequently absent. Thus the fall in arterial

TABLE I

COMPARISON OF THE ANALGESIC ACTION OF BENZETHIDINE, FURETHIDINE AND PETHIDINE IN RATS

The limits of error (P=0·95) of the ED50's are shown in parentheses.

Compound	Subcutaneous			Oral		
	ED50 mg./kg.	Duration (min.)	Relative Potency	ED50 mg./kg.	Duration (min.)	Relative Potency
Benzethidine	0·92 (0·48–1·76)	60	8	12·0 (10·0–38)	115	3
Furethidine	0·45 (0·27–0·65)	60	16	2·8 (1·8–4·4)	130	13
Pethidine	7·2 (3·0–12·5)	60	1	37·7 (23·4–45·6)	90	1

TABLE II

COMPARISON OF THE EFFECTS OF BENZETHIDINE, FURETHIDINE AND PETHIDINE ON RESPIRATION

The relative potencies were calculated from the reduction in respiratory rate of rats, and from measurements of minute volume in the rabbit, cat and dog.

	Relative Potencies			
	Unanaesthetized Rats (Subcutaneous)	Anaesthetized Rabbit (Intravenous)	Cat (Intravenous)	Dog (Intravenous)
Pethidine ..	1	1	1	1
Benzethidine ..	2·8 (1·6–5·6)	5	3	5
Furethidine ..	24·3 (12·8–45·3)	16	8	10

TABLE III

INCREASE IN HEXOBARBITONE-INDUCED SLEEPING TIME

Subcutaneous injection in mice.

Drug	Dose (mg./kg.)	Increase in Sleeping Time (%)
Benzethidine	5	63
Furethidine	1	130
Pethidine	20	265

blood pressure caused by pethidine was more rapid and long-lasting than that caused by benzethidine or furethidine.

A slight reduction in heart rate was observed with higher doses of all three drugs, but no changes in the electrocardiogram were observed. The drugs had no effect on the responses of the vascular system to injected adrenaline, noradrenaline, acetylcholine, histamine, nicotine or vasopressin.

Behaviour and Activity.—In doses producing analgesia, no sedative or hypnotic effects were seen in the mouse, rat, cat, dog or monkey. Higher doses of furethidine (4 mg./kg.), benzethidine (20 mg./kg.), pethidine (40 mg./kg.), that is, up to 10 times the analgesic doses, produced sedation in all animals except the cat which showed a pattern of excitation similar to that caused by morphine ; lower doses produced no excitement.

No reduction in activity in mice made hyperkinetic with $\beta\beta$-iminodipropionitrile was observed in analgesic doses ; higher doses reduced the degree of hyperactivity although the animal still responded normally to nociceptive stimuli.

Antitussive Action. — All three compounds prevented artificially induced cough in anaesthetized cats: the minimal doses needed to abolish cough by intravenous injection were benzethidine, 0.5 mg./kg. ; furethidine, 0.1 mg./kg. ; and pethidine, 2.0 mg./kg.

Prolongation of Barbiturate-induced Sleep.—When given in doses producing equal analgesic effects, benzethidine and furethidine produced less prolongation of hexobarbitone-induced sleep than did pethidine (Table III).

TABLE IV

RELATIVE POTENCIES OF BENZETHIDINE AND FURETHIDINE (PETHIDINE=1) IN DIFFERENT TESTS

Compound	Mydriasis	Local Anaesthesia	Charcoal Meal Test	Inhibition of Peristaltic Reflex	Antagonism of Barium Chloride	Histamine Liberation	Analgesia
Benzethidine ..	1	3·5	5	6	0·6	0·15	8
Furethidine ..	20	3·5	10	14	32	0·02	16

Anticonvulsant Effect.—In doses twice the analgesic ED50, none of the three compounds showed any protective effect against leptazol-induced convulsions in mice.

Emetic Action.—Merlevede and Levis (1958) claim to produce vomiting in 50% of dogs with an intramuscular injection of 10 mg./kg. of pethidine hydrochloride. No emesis was observed in dogs with benzethidine 2 mg./kg. injected intramuscularly or 5 mg./kg. orally or with furethidine 0.5 mg./kg. injected intramuscularly or 1 mg./kg. orally, but 10 mg./kg. of pethidine injected intramuscularly produced emesis in one dog out of three.

Pupillary Action. — Benzethidine, furethidine and pethidine produced mydriasis in the mouse, rat and cat, but myosis in the dog. The potency ratios are shown in Table IV.

Local Anaesthetic Action.—Both benzethidine and furethidine possess local anaesthetic properties and are approximately 3 times more potent than pethidine and procaine (Table IV). All 4 compounds had a duration of action of approximately 30 min. in the guinea-pig. When given intradermally in man, furethidine and benzethidine had a local anaesthetic action which lasted about 60 and 45 min. respectively.

Histamine-releasing Action. — Pethidine, like morphine, has been shown by previous workers to be capable of releasing histamine. When benzethidine and furethidine were compared with pethidine in doses producing an equal analgesic effect a striking difference in the histamine-releasing properties was apparent. Furethidine and normal saline produced weals of the same size and the amount of histamine released by benzethidine was very small.

Table IV shows the relative histamine-releasing properties of the three compounds as measured by the weal area produced by approximately equi-analgesic doses. The plot of weal area against log dose gave parallel lines.

Gastro-intestinal Effects.—In common with pethidine, benzethidine and furethidine showed a general, apparently non-specific depressant effect on smooth muscle. All three drugs inhibited the normal spontaneous contractions of isolated rabbit duodenum. They also antagonized the stimulant actions of acetylcholine, histamine and barium chloride on the guinea-pig ileum.

TABLE V

ACUTE TOXICITY OF BENZETHIDINE, FURETHIDINE AND PETHIDINE

The figures represent LD50s (mg./kg.); limits of errors (P=0·95) shown in parentheses.

	Mice (Intravenous)	Rats (Subcutaneous)	Rats (Oral)
Benzethidine ..	10·9 (10·1–11·5)	600 (400–900)	284 (57–460)
Furethidine ..	15·5 (14·1–17·1)	26·2 (14·5–47·1)	135 (125–146)
Pethidine ..	58·5 (54·2–63·1)	360 (334–388)	760 (510–1,140)

TABLE VI

THE EFFECT OF BENZETHIDINE, FURETHIDINE AND PETHIDINE ON THE GROWTH RATE OF RATS

Each drug was injected intraperitoneally in a group of 20 male rats and compared with a similar group injected with saline.

Drug	Dose (mg./kg.)	F Ratio	*P*
Benzethidine ..	5	1·4	>0·10
Furethidine ..	1	1·0	>0·50
Pethidine	25	1·1	>0·25

Like all potent analgesics benzethidine and furethidine inhibited peristalsis in the isolated guinea-pig ileum preparation. All three drugs given subcutaneously reduced the quantity of faeces passed by normal rats. The potency ratios are given in Table IV.

Antidiuretic Action.—In common with all other potent analgesics tested pethidine, benzethidine and furethidine showed antidiuretic properties when injected intravenously in rats.

The antidiuretic responses of different rats of 200 g. body weight showed a wide variation ; the total doses of drug to produce a 40% inhibition of urine output were benzethidine 11 μg., furethidine 30 μg., and pethidine 100 μg.

The antidiuresis appears to be due to the release of antidiuretic hormone as no antidiuretic response was observed during a saline diuresis.

Acute Toxicities.—Table V shows the acute toxicities for benzethidine, furethidine and pethidine. The toxic effects of benzethidine and furethidine appeared to be a result of depression of the central nervous system ; pethidine, on the other hand, produced excitation and clonic convulsions prior to death, symptoms not seen with benzethidine and furethidine.

Subacute Toxicities.—None of the drugs tested had any significant influence on the growth rate of male albino rats, when compared with a control group of rats treated with saline (Table VI). The mortality rate in the treated groups was not significantly different ($P>0.1$) from that of the control group.

No pathological changes attributable to the administration of the drugs were detected in any of the internal organs ; slight necrotic areas were apparent at the injection site in 3 rats treated with pethidine and 2 from the group treated with benzethidine ; no lesions were seen in the furethidine or the control groups.

Haemoglobin concentration, red cell count and differential white cell count were all within normal limits.

Tanabe and Cafruny (1958) have suggested that the development of tolerance to the analgesic action of morphine is accompanied by adrenal hyperplasia ; in the present investigation, however, no significant difference ($P>0.1$) was found between the adrenal weights of the control and the treated groups.

Discussion

For many years it was believed (Braenden, Eddy, and Halbach, 1955) that for high analgesic potency the optimum substituent on the nitrogen atom in the pethidine series was a methyl group ; within the last four years, however, many norpethidine derivatives with markedly higher potency than pethidine have been prepared. Little advantage is to be gained by increasing the potency of an accepted drug if this increase is paralleled by an increase in the incidence of undesirable side-effects. Present research is therefore aimed at achieving a reduction in side-effects caused by a drug administered at an adequate analgesic dose.

Millar and Stephenson (1956) and Blair and Stephenson (1960) have produced evidence that the dose-response curves for analgesia of a number of substituted norpethidine derivatives are not parallel with that of pethidine. Pethidine is an inadequate analgesic for severe pain, because increase of the dose above a certain level does not produce a corresponding increase in analgesia, that is, a plateau in the dose-response curve is reached. As the dose-response curves for furethidine and benzethidine appear to be steeper than that of pethidine a greater degree of analgesia may be possible with these drugs than with pethidine.

The increased analgesic potency of benzethidine and furethidine compared with pethidine appears to be accompanied by an increase in respiratory depression. Respiratory depression, however, only occurs in doses which are higher than those producing an adequate level of analgesia. Since benzethidine and furethidine are considerably more potent than pethidine, it may be possible to control severe pain with these drugs without causing excessive respiratory depression.

In equi-analgesic doses benzethidine and furethidine produce less potentiation of barbiturate-induced sleep in mice than does pethidine ; this indicates that the latter drug may produce more general central nervous depression than benzethidine and furethidine. Reduced sedation has been found particularly useful in obstetric analgesia when it is desirable that the patient should remain unsedated and fully co-operative.

An interesting finding is that benzethidine and furethidine on intradermal injection release much less histamine from the skin than does pethidine in doses of equal analgesic potency. Gershon and Shaw (1958) have suggested that a number of the undesirable side-effects of morphine are due to its histamine-releasing properties and that many of these effects can be controlled by the administration of an antihistamine ; thus it is possible that benzethidine, and furethidine in particular, may

show a decreased liability to produce these troublesome side-effects. In the limited experiments using dogs, no side-effects attributable to histamine release were produced by benzethidine and furethidine in effective analgesic doses.

In anaesthetized cats the fall in blood pressure which follows the intravenous injection of pethidine is greater than that with benzethidine and furethidine ; part of this hypotensive response can be blocked by the administration of an antihistamine drug, and this lends further support to the finding that benzethidine and furethidine are less potent histamine liberators than is pethidine.

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THE EXCRETION AND STABILITY TO METABOLISM OF BRETYLIUM

BY

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Bretylium (*o*-bromobenzylethyldimethylammonium) is a new type of hypotensive drug. It was estimated in extracts of human urine as the methyl orange complex. From 7 to 45% of a single oral dose was found in human urine within 9 hr. The [^{14}C] labelled drug was used to investigate excretion by cats. A minor proportion of a subcutaneous dose was eliminated by cats in the faeces, probably after secretion into the bile. Most of the dose was excreted unchanged in the urine. No products of metabolism were found in either human or cat urine. The drug suffered negligible change when incubated with rat liver tissue *in vitro*.

Bretylium tosylate (Darenthin ; *o*-bromobenzyl-ethyldimethylammonium toluene - *p* - sulphonate) is a quaternary salt described by Boura, Copp, and Green (1959) that selectively impairs the function of adrenergic neurones and lowers the blood pressure of hypertensive patients (Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim, 1959). It is notable for its freedom from central effects and for lack of actions on the parasympathetic nerves which mar the use of ganglion blocking agents. For a quaternary salt it is fairly well absorbed as judged by its effects in both man and animals. By the same criterion its absorption by any one patient was found to be fairly constant, but there was considerable variation in the effective dose needed by different patients. This paper gives information on the excretion of the drug and its stability to metabolism.

METHODS

Assay of Bretylium in Human Urine.—A satisfactory specific method of chemical assay was not devised ; bretylium is stable to chemical reagents under mild conditions and lacks physical properties suitable for assay purposes. Moreover, the drug is very hydrophilic, its reineckate rather soluble (about 20 mg./l.) and continuous extraction very slow. The following two methods, based on the well-known association of bases with sulphonic acid dyes, were intended to provide approximate values for assessing the excretion of the drug in human urine.

A preliminary estimate was gained by chromatography of 100 μl. portions of urine in the organic phase of n-butanol/acetic acid/water (4:1:5). The size of the spot revealed at Rf 0.75 by spraying with Dragendorff's reagent was compared with standard spots of 2 to 10 μg. of the cation. Rf values in other solvent mixtures were 0.75 in s-butanol/acetic acid/water (12:5:3) and 0.67 in n-butanol/pyridine/water (1:1:1).

Method A.—The sample was diluted with an equal volume of ethanol and filtered. An aliquot of the filtrate, usually 2 ml., was diluted with 5 vol. of 0.05M phosphate buffer, *p*H 7, and the solution passed down a column (10 × 1 cm.) of Amberlite IRC-50 resin buffered at *p*H 7. The column was washed to *p*H 5 by 300 ml. 0.05M phthalate buffer, and the eluate, containing interfering bases, rejected. Bases retained on the column were eluted by 150 ml. warm 0.5N hydrochloric acid. The eluate was neutralized, evaporated under reduced pressure, and extracted with 3 × 20 ml. portions of ethanol. Ethanolic methyl orange (0.05%, 3 ml.) was added, and the solution dried under reduced pressure. The base-dye complexes were extracted with several portions of warm ethylene dichloride until the washings were colourless. The extract was adjusted to volume, usually 25 ml., and the red colour produced by dilution with an equal volume of ethanolic hydrochloric acid (1%) was measured at 525 mμ.

Recovery of 1 mg. amounts of bretylium iodide from normotensive urine averaged 98 ± 11% in four trials. A major source of error arose from colloidal methyl orange carried over in the ethylene dichloride if evaporation of water was not sufficiently thorough. The lengthy procedure could be shortened for urines containing more than 10 mg. of drug/100 ml. (as shown by chromatography). The neutralized acid eluate from the resin was evaporated to a few ml. and 1 ml. 0.5N sulphuric acid and 5 ml. sodium

phosphate (5%) added. A blue complex was formed after shaking with 10 ml. of a cobalt thiocyanate reagent (Ashbrook, 1959); this complex was extracted by thorough shaking with 10 ml. ethylene dichloride. Emulsions were troublesome. The blue colour was read at 620 mμ.

Method B.—In single-dose experiments, blank values were obtainable from urine specimens collected from the patients at the beginning and end of the experiment and it was convenient to obtain a result rapidly by distilling 1 to 2 ml. of the sample with 3 ml. ethanolic methyl orange (0.05%) and 50 ml. ethylene dichloride, until the distilling temperature had reached 85°, adding more solvent if necessary. The solution in the flask was cooled, filtered, adjusted to 25 ml. and read as in Method A. Recovery of 1 mg. amounts of drug from normotensive urine averaged 92±13% in three trials.

Assay of [^{14}C]-labelled Bretylium. — Urines in appropriate amounts were dried on lens paper on planchettes and counted under an end window counter. The results were referred to standards prepared by adding known amounts of labelled drug to similar urines. Faeces were digested with hot 2N hydrochloric acid, cooled and filtered. The volume was adjusted to 100 ml. and aliquots counted as described for urine. Similar methods were used in some experiments with tissues *in vitro*, but where greater accuracy was desired or where the level of radioactivity was low, the sample was dried and combusted in oxygen, with added glucose if necessary, and the carbon dioxide was counted as gas (Glascock, 1954).

Identity of the Excreted Material.—The mobilities of spots revealed by Dragendorff's reagent and autoradiography of paper chromatograms of whole urines were compared in the solvent systems mentioned above with authentic material dissolved in the same samples.

Attempts to isolate pure specimens of bretylium iodide or bromide after precipitation of urinary bases as the reineckates were unsuccessful, and an isotope dilution method was applied. A cat received 22 mg. of [^{14}C]-labelled bretylium iodide subcutaneously. The 1 to 2 and 3 to 5 day urines were collected separately, made up to volume, and aliquots retained for [^{14}C] assay. Carrier bretylium bromide (400 mg.) was added to each sample followed by evaporation to dryness and extraction with ethanol. A portion of each extract was retained for [^{14}C] assay before evaporation and precipitation of the reineckates. These were crystallized once from dilute acetone, and the bases were liberated by silver sulphate. The bases were chromatographed on Whatman 3 MM paper in n-butanol/acetic acid/water. The region indicated on small strips sprayed with Dragendorff's reagent was eluted with water and the eluate evaporated. Bretylium was finally isolated as the reineckate for analysis of [^{14}C] by combustion to carbon dioxide and counting as gas. The reineckates were crystallized from dilute acetone, m.p. 175–178° (decomp.). (Found: C, 32.0; H, 4.0; N, 17.5. $C_{15}H_{23}N_7S_4CrBr$ requires C, 32.1; H, 4.1; N, 17.5%).

Search for Possible Products of Metabolism of Bretylium

Carbon Dioxide.—A rat (100 g. wt.) received 1 mg. of the labelled drug subcutaneously. It was kept in a metabolism chamber in a slow stream of air, and the issuing gas was scrubbed in two towers containing glass beads moistened with 5N potassium hydroxide. Trapped carbon dioxide was liberated into a vacuum line and counted as gas.

Bromide Ion.—A portion of a 24 hr. specimen of urine from a patient who had received 2,250 mg. of bretylium tosylate the day before was examined for bromide ion by the method of Belote (1927).

o-Bromobenzoic Acid.—A 24-hr. specimen of urine from a patient who was receiving 900 mg. of bretylium iodide daily was evaporated to a syrup and hydrolysed by concentrated hydrochloric acid for 5 hr. at 100°. The solution was diluted to 250 ml. and extracted with ether. Organic acids were dissolved out by alkali and transferred back to ether. Removal of the solvent gave 450 mg. of dark material which yielded 350 mg. of white crystals, m.p. 153 to 156°, to hot ligroin. Neither these nor the crude material contained bromine. The crystals were identified by conventional methods as salicylic acid.

Experiments with Liver Tissue in vitro.—Rat liver slices were incubated for 2 to 3 hr. in Krebs-Ringer glucose phosphate saline containing [^{14}C]-labelled bretylium iodide (10^{-3} or 2×10^{-4}M). The rate of oxygen uptake, 46 μ-moles/g. tissue/hr., did not differ from similar experiments in which bretylium was omitted. Evolved carbon dioxide was recovered either from the pooled filter papers containing potassium hydroxide from the centre wells of manometric vessels or from baryta used to wash the issuing gas from aerated mixtures. Trichloroacetic acid extracts of homogenized incubation mixtures were chromatographed in n-butanol/acetic acid/water for autoradiography. The spots on chromatograms, corresponding to controls where the drug was added after completion of incubation, were excised and counted directly on the paper by a scintillation technique (Roucayrol, Oberhauser, and Schussler, 1957) using a non-volatile scintillator (Buck and Swank, 1958).

Liver homogenates were incubated in the fortified medium containing semicarbazide to trap formaldehyde as described by Axelrod (1956). Any formaldehyde produced during metabolism was distilled into phenylsemicarbazide solution for combustion and counting.

RESULTS

Excretion by Cats.—Estimation of the radioactivity in cat urine after subcutaneous doses of [^{14}C]-labelled bretylium iodide (10 mg./kg.) showed

TABLE I

EXCRETION OF [^{14}C]-LABELLED BRETYLIUM IN CATS

Cats were injected subcutaneously with bretylium tosylate 10 mg./kg. Figures are % of dose excreted. Serial estimations in single cats except the first row of urine figures and the bile figures.

	Hours			Days						
	1	12	18	1	2	3	4	5	6	7
Urine	15	30	74	—	—	—	—	—	—	—
,, ...	—	—	—	46·5	10·2	5·7	4·2	1·3	1·7	1·0
,, ...	—	—	44·7	—	14·1	4·7	—	—	—	—
Faeces	—	—	0	0·5	5·5	6·4	0·2	—	—	—
,, ...	—	—	—	—	1·5	—	—	5	—	—
Bile	1·3	0·5	0·1	—	—	0·006	—	—	—	—

that excretion began within 1 hr. and that about half the dose was excreted by this route within 24 hr. (Table I). In one cat, 83% of the dose was accounted for in urine and faeces within 7 days, though detectable radioactivity was still present in urine on the seventh day. The drug was secreted into bile and this may account for the minor amounts found in the faeces after the subcutaneous dose. In another experiment, in which 50 mg./kg. of bretylium iodide was given subcutaneously, 12.7 mg. was found in the bile after 18 hr.

Excretion in Human Urine.—Because of the unsatisfactory nature of the assay used, the results shown in Tables III and IV can only be regarded as approximations. The standard deviations in recovery experiments from normotensive urines were rather high, but the greatest source of error arose from the high blanks given by urines from hypertensive subjects. Normotensive subjects appeared to excrete less basic material, and their urines by Method B gave values that ranged from the equivalent of 1.4 to 8.3 μg./ml. bretylium tosylate (mean of 6 experiments=4.1 μg./ml.). However, one further specimen of urine from a normotensive subject gave a value equivalent to 167 μg./ml. bretylium tosylate. Urines from 4 hypertensive subjects gave values equivalent to 50, 158, 214, and 368 μg./ml. These high values could not be traced to previous drug regimens. The resin treatment of Method A reduced these values to an equivalent of between 8 and 32 μg./ml. bretylium tosylate.

Table II gives a comparison of the values obtained by three variants of the methyl orange method on the same series of specimens. The third set of figures refers to the application of Method B to the eluate after an initial purification by chromatography. The figure for the second day by Method A appears to be high, and recovery of small amounts of drug from paper was unsatisfactory, otherwise there was reasonable agreement considering the very high blank values given by this patient. High blank values were also found in a specimen from the same patient before bretylium treatment. The result suggests that about one third of the total dose was excreted in urine by this patient.

TABLE II

COMPARISON OF METHODS FOR ESTIMATING BRETYLIUM IN URINE

24 hr. urine specimens were collected on 5 successive days from a patient. During the first 2 days the patient received 900 mg. bretylium iodide and for the last 3 days a placebo only. The specimens were assayed by 3 different methods. Each figure represents the total 24 hr. excretion in mg. of total bases or bretylium iodide, and is the mean of two determinations. Total bases were calculated in terms of bretylium iodide. The mean of days 4 and 5 was taken as the blank for calculating the amount of bretylium excreted.

Day No.	Method A		Method B		Method B with Chromatography	
	Total Bases	Bretylium Iodide	Total Bases	Bretylium Iodide	Total Bases	Bretylium Iodide
1	980	505	595	496	448	424
2	707	232	216	117	127	103
3	536	61	168	69	20	0
4	456	—	106	—	24	—
5	494	—	92	—	—	—
Total		798		682		527

Table III shows that excretion of single oral doses reached a peak at 2 to 4 hr. after the dose, and that excretion had virtually ceased after 9 hr. The degree of absorption by different patients was variable. In the one instance studied (Table III), total absorption did not appear to be influenced by a heavy meal though the scatter of the figures after a meal contrasts with the smooth rise and fall seen in the same patient when fasting. Table IV shows excretion of total bases while on continued dosage with bretylium; it indicates that excretion became appreciable only when the total daily dose had exceeded 1 g. The very high value for the 15th day (confirmed by chromatography) shows that sudden absorption may occur for no apparent reason.

Metabolism in Vivo.—Chromatograms and autoradiograms of urine and bile from cats that had received the labelled drug showed spots with the same mobilities as bretylium. Trichloroacetic acid extracts of heart, spleen, lung, and superior cervical ganglion also contained unchanged drug. Examination of these chromatograms, and numerous chromatograms of whole human urine, by ultraviolet light and conventional spray reagents, failed to disclose anything that could be interpreted as a metabolic product of the drug.

A rat that had received labelled bretylium produced radioactive carbon dioxide equivalent to 0.03% of the dose during 6 hr. but none in the next 17 hr. No bromide ion was found in the human urine examined.

An isotope dilution method failed to disclose the presence of metabolic products in the urine of a cat. The reineckate was used as a convenient means of purifying the bretylium. The dilution factor calculated from estimation of [^{14}C] in the purified reineckate did not exceed that calculated from estimation of total [^{14}C] in an alcoholic extract of the urine. For a 1 to 2 days sample, the reineckate and alcohol extract factors were 113 and 116 respectively, and for the 3 to 5 days samples, 137 and 163.

Metabolism in Vitro.—Rat liver slices suspended in saline containing labelled bretylium evolved small amounts of labelled carbon dioxide at a rate of 0.15% added drug/g. tissue/hr. The result parallels the slow rate of evolution of carbon dioxide by the rat after dosage with the labelled drug. Attempts to detect formaldehyde such as could arise by *N*-demethylation were negative. Recovery of labelled drug after incubation with liver tissue was assessed by chromatography of protein-free extracts and

TABLE III

URINARY EXCRETION OF BRETYLIUM DURING A 24 HR. PERIOD

Patients were given oral doses of bretylium at 9 a.m. Thereafter urine samples were collected at intervals and assayed for total bases. Values represent mg./hr. of total bases in terms of bretylium. The % excretion was calculated as the total bases excreted from 9 a.m. to 6 a.m., less the mean of the 6 a.m. to 9 a.m. and 6 p.m. to 5 a.m. values. I=bretylium iodide, T*=bretylium tosylate with a heavy meal. T†=bretylium tosylate in the same patient while fasting.

	Method B			Method A	
Dose:	500 mg. I	300 mg. I	500 mg. I	500 mg. T*	500 mg. T†
Time	mg./hr.	mg./hr.	mg./hr.	mg./hr.	mg./hr.
6 a.m.– 9 a.m. . .	1·2	5·5	2·5	8·8	2·4
9 ,, 10 ,,	41·3	7·3	12·0	10·6	3·8
10 ,, 11 ,,				5·5	6·2
11 ,, –12 noon				26·4	9·8
12 noon –1 p.m.	41·5	21·3	7·3	3·5	7·3
1 p.m.– 3 ,, . .	24·3				
3 ,, – 6 ,, . .	6·8	13·3	9·0	8·0	5·3
6 ,, – 5 a.m. . .	1·3	2·4	3·3	1·0	1·9
% of dose excreted	45%	30%	12%	7%	8%

TABLE IV

URINARY EXCRETION OF TOTAL BASES BY TWO PATIENTS ON CONTINUED DOSAGE WITH BRETYLIUM TOSYLATE

The total bases were assayed by Method A and expressed in terms of bretylium tosylate, mg./24 hr.

Day	Dose	Total Bases	Dose	Total Bases
1	—	12·6	—	—
2	—	10·3	—	20·5
3	375	15·4	—	—
6	750	19·5	875	30·6
7	1,000	13·2	1,125	40·0
8	1,375	36·8	1,125	34·9
9	1,750	43·6	1,500	33·2
14	2,250	49·2	—	—
15	2,250	285·0	—	—
16	2,250	61·5	—	—
17	2,250	40·0	—	25·6
18	2,250	61·5	—	8·3
20	2,250	79·2	—	7·1

counting the appropriate areas of paper directly. The chromatograms from incubated mixtures gave $1,690 \pm 265$ counts/min., those from mixtures where labelled bretylium was added after incubation $1,600 \pm 160$ counts/min. (means of 6 results).

Discussion

No evidence was obtained to suggest that bretylium is metabolized in the body to any marked extent, though evolution of labelled carbon dioxide by rats and from liver incubated with the drug indicates that it is not completely immune from metabolic attack. Complete oxidation or extensive debromination by a dehalogenase would have been revealed by presence of bromide ion in urine. The evidence from chromatography and autoradiography can only apply to products with the quaternary group intact, but removal of this group would be expected to give a benzoic acid. The salicylic acid found in one urine almost certainly arose from ingested acetylsalicylic acid. Metabolites that retain the quaternary nitrogen such as a hypothetical mercapturic acid seem to be unlikely since isotope dilution give no hint of other products. Substituted bromobenzenes do not give rise to mercapturic acids (Williams, 1947).

Compared with many other quaternary salts, bretylium is absorbed fairly readily from the alimentary tract. In unpublished work it has been shown to be absorbed from the intestine and not from the stomach, but the mechanism is obscure. Current theories on drug absorption, that assume solution of unionized bases in lipid, are difficult to apply to quaternary salts, unless they form an unionized complex analogous to the complexes with sulphonic acid dyes, with some constituent of the intestine.

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THE SELECTIVE ACCUMULATION OF BRETYLIUM IN SYMPATHETIC GANGLIA AND THEIR POSTGANGLIONIC NERVES

BY

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A study of the distribution of [^{14}C]-labelled bretylium (*N*-*o*-bromobenzyl-*N*-ethyl-*N*,*N*-dimethyl-ammonium) in cat tissues at various times after subcutaneous injection suggests that the specificity of its blocking action on adrenergic neurones may be related to its selective accumulation in these neurones. The rate of rise and fall of concentration in sympathetic ganglia and postganglionic sympathetic nerves showed a close similarity to the time course of the blocking adrenergic neurones as manifested by relaxation of the nictitating membranes. Concentrations found were similar to those in adrenergic nerve trunks when topical application of the drug had caused a local block of conduction. Conduction in other types of nerve could be blocked by topical application, but in general they were less sensitive, heavily myelinated nerves being the most resistant.

The pharmacology of bretylium (*N*-*o*-bromobenzyl-*N*-ethyl-*N*,*N*-dimethyl ammonium) has been described by Boura and Green (1959). The drug impaired the function of adrenergic neurones in doses which did not affect the parasympathetic or central nervous systems. It has been successfully used to control hypertension in man (Boura, Green, McCoubrey, Laurence, Moulton, and Rosenheim, 1959; Smirk and Hodge, 1959; Dollery, Emslie-Smith and McMichael, 1960). This study of the distribution of the [^{14}C]-labelled drug in cats suggests that the specificity of action of bretylium may be related to its selective accumulation and retention in adrenergic neurones.

METHODS

N-^{14}C-methyl Labelled Bretylium Iodide.—Methyl iodide, containing a nominal 3 mC of [^{14}C] (0.77 m.mole), was condensed *in vacuo* on to 0.77 m.mole of *N*-o-bromobenzyl-*N*-ethyl-*N*-methylamine in 1.8 ml. methyl acetate. The mixture was sealed off and allowed to stand at room temperature for 24 hr. The product separated as an oil that crystallized on standing. It was filtered off, washed with methyl acetate, and used without further purification (m.p. 119 to 120° on a hot stage; yield, 219 mg.). The material, suitably diluted with inactive carrier, was combusted in oxygen and [^{14}C] was counted as carbon dioxide (Glascock, 1954). It was calculated that the undiluted material had a specific activity of 3.1 mC/m.mole≡79% of the [^{14}C]-methyl iodide used. Paper chromatography in several solvent systems followed by autoradiography revealed one radioactive spot at the position stained brown by Dragendorff's reagent. R_F values were as follows: in *s*-butanol-acetic acid-water (12:5:3), 0.75; in *n*-butanol-pyridine-water (1:1:1), 0.67; and in *n*-butanol-ethanol-10% ammonia (40:20:13), 0.58. This material was diluted with two or three parts of unlabelled bretylium iodide before use.

Tissue Sampling After Subcutaneous Administration of Bretylium Iodide.—For most experiments cats received 10 mg./kg. in saline. After a suitable time interval they were anaesthetized with ether and bled out by cutting the aorta. The blood was collected and heparin added. Tissues were dissected as rapidly as possible, taking the nerves and ganglia first, then samples of larger organs, and finally samples of the central nervous system. The dissection took about 1 hr.

Ganglia, nerves and other small samples were rapidly trimmed on filter paper, weighed on a torsion balance to 0.5 mg., and laid in porcelain boats for drying at 100°. Larger samples were weighed into tared tubes and dried over phosphorus pentoxide *in vacuo* for two days.

Topical Application to Nerve Trunks.—Labelled bretylium was applied topically to several nerve

trunks in cats under chloralose anaesthesia, in order to determine its uptake by the nerves and its effect on conduction. The latter was assessed from the response of a suitable end organ when the nerve was stimulated proximal to the portion exposed to the drug. The following nerves were examined:

(*a*) Inferior cardiac: the heart rate was followed with a Cushny myocardiograph triggering a Thorp impulse counter.

(*b*) Hypogastric: the relaxant effect of nerve stimulation on the uterus was recorded by means of a Cushny myocardiograph.

(*c*) Preganglionic cervical sympathetic: the end organ response was the contraction of the nictitating membrane, recorded isotonically with a frontal writing lever.

(*d*) Greater splanchnic: carotid blood pressure was recorded with a mercury manometer. Conduction in the nerve was considered to be impaired when the pressor effect of nerve stimulation was decreased.

(*e*) Phrenic: isometric contractions of the diaphragm were recorded.

In each of these preparations the nerve was cut and freed from surrounding tissue for a length of 2 to 3 cm., and then drawn through a glass chamber with rubber dam seals at each end. This was filled with Tyrode. The proximal end of the nerve, under warm liquid paraffin, was stimulated through platinum electrodes with supramaximal shocks at 15/sec., for periods of 30 sec. every 3 or 5 min. When the response of the end organ was constant, the fluid in the nerve bath was replaced with Tyrode containing labelled bretylium. At the times stated in Table III when the end organ response, usually diminished, was again fairly constant, the immersed segment of nerve was excised, quickly rinsed in Tyrode, blotted, weighed and dried at 100° ready for assay. In one experiment the superior cervical ganglion and the adjacent portions of its pre- and post-ganglionic nerves, freed from connective tissue but otherwise intact, were immersed in a small plastic

TABLE I

THE CONCENTRATION OF BRETYLIUM IN PERIPHERAL NERVOUS TISSUES

Values for individual cats at intervals following subcutaneous injection of 10, 6 or 50 mg./kg. of [^{14}C]-labelled bretylium iodide. Concentrations are expressed as mμmoles/g. wet tissue. 1 mμmole=0·37 μg. bretylium iodide.

Tissue	10 mg./kg.					6 mg./kg.	50 mg./kg.
	3 hr.	12 hr.	18 hr.	18 hr.	72 hr.	18 hr.	18 hr.
Adrenergic nerves							
Postganglionic cervical sympathetic	42	78	—	700	31	49	696
Hypogastric	—	174	—	—	41	61	421
Gastric	—	99	—	—	—	62	—
Inferior cardiac	22	136	—	—	19	63	196
Cholinergic nerves							
Preganglionic cervical sympathetic	28	29	—	80	8	23	114
Greater splanchnic	14	30	—	—	8	24	37
Vagus	15	19	—	40	2	—	47
Phrenic	11	94	—	40	—	—	—
Sympathetic ganglia							
Superior cervical	197	270	445	890	61	174	965
Middle cervical	—	—	—	610	—	—	—
Stellate	168	315	303	770	16	185	728
Coeliac	158	374	450	950	41	225	634
Superior mesenteric	—	59	258	700	—	—	—
Inferior ,,	290	362	—	430	49	152	440
Other ganglia							
Nodose	38	15	41	30	1	6	37
Ciliary	—	64	—	30	—	—	—
Otic	—	7	—	1	—	—	—
Dorsal root	—	16	—	10	—	—	—
Semilunar	—	<1	—	1	—	—	—

TABLE II

THE CONCENTRATION OF BRETYLIUM IN VARIOUS TISSUES

Values for individual cats at intervals after subcutaneous injection of 10 or 50 mg./kg. of [^{14}C]-labelled bretylium iodide. Concentrations are expressed as mμmoles/g. wet tissue. 1 mμmole=0·37 μg. bretylium iodide. (a) Other tissue concentrations in this cat were: parotid gland 20 mμmole, ovary 38 mμmole, thyroid 14 mμmole, cervical lymph gland 5 mμmole; (b) and (c) are the concentrations in the cortex and medulla respectively; (d) left ventricle; (e) cerebral and cerebellar cortex and spinal cord.

	10 mg./kg.							50 mg./kg.
	1 hr.	3 hr.	3 hr.	12 hr.	18 hr. (a)	18 hr.	72 hr.	18 hr.
Blood	23	15	—	13	28	17	<1	24
Liver	288	78	201	62	23	27	—	—
Kidney	113	—	—	—	9	13	—	—
Adrenal gland ..	77	53	—	20	78	110 (b) 164 (c)	<1	85
Heart (d)	400	131	146	44	22	32	<1	38
Spleen	205	34	102	235	142	108	25	45
Lung	—	88	—	—	—	—	—	—
Diaphragm ..	52	—	—	—	45	50	—	—
Area postrema ..	1	25	—	—	—	20	—	—
C.N.S. (e)	<1	<1	—	<1	—	<1	—	—
Hypothalamaus ..	1	3	—	—	—	—	—	—

trough. Stimulation was applied to the preganglionic nerve. After immersion in bretylium, the concentrations in the ganglion and in the immersed portions of the pre- and the postganglionic trunks were assayed separately.

Assay.—The small samples were combusted in oxygen and [^{14}C] counted as carbon dioxide (Glascock, 1954). Most of the nerve samples weighed between 7 and 10 mg. Where necessary, inactive glucose was added to bring the total weight within this range. From the measured volume of carbon dioxide produced and the specific activity, the total activity in each sample was calculated. Large samples were dried *in vacuo* over phosphorus pentoxide for several days, powdered, and plated out on polythene planchettes for counting at infinite thickness under an end window counter.

The results were calculated by reference to calibrations prepared from samples containing known amounts of activity. Most counts were taken to within 2% standard deviation, but samples of low activity had standard deviations up to ±10%.

RESULTS

Previous experiments (Boura and Green, 1959) have shown that the nictitating membranes of cats injected subcutaneously with 10 mg./kg. bretylium bromide began to relax after 3 hr., were fully relaxed at 12 and 18 hr., and regained their normal tone only after two to three days. The time course of this effect, found also in the present series of experiments with the labelled bretylium iodide, was characteristic of the slow and persistent adrenergic neurone blocking action of the drug. It was in relation to this that the distribution of bretylium at various time intervals after injection (Tables I and II) is of particular interest.

The levels of radioactivity found in sympathetic ganglia and their postganglionic trunks showed that these tissues attained far higher concentrations of the drug than did any others examined (Tables I and II). At 18 hr. after subcutaneous injection of 10 or 50 mg./kg., when the nictitating membranes were fully relaxed, these ganglia contained as much as 250 to 1,000 mμmoles/g., and their postganglionic trunks 200 to 700 mμmoles/g. The rate of accumulation of these concentrations was slow, especially by the nerve trunks. The values at 72 hr. indicate that the rate of decline was also slow. The other nerves and ganglia examined accumulated moderate concentrations, in contrast to the high concentrations found in adrenergic nerves and sympathetic ganglia.

The concentration of bretylium in blood and in some other organs is shown in Table II. One hour after injection the blood contained about 20 mμmoles/ml., and similar levels were found at 3, 12 and 18 hr., but much less at 72 hr. Differential centrifugation of the blood in siliconed tubes showed that the concentration was

approximately the same in plasma and red cells. There was no more bretylium in the platelet fraction than could be accounted for by residual plasma. Other organs, for example, liver, kidneys, spleen and heart, accumulated concentrations far exceeding that in the blood within 1 hr. Levels in most organs had greatly declined within 12 to 18 hr., but those in the adrenal gland and spleen were well maintained. No drug was found in the central nervous system except for small amounts in the hypothalamus and area postrema.

After a dose of 6 mg./kg. only partial relaxation of the nictitating membranes occurred; 18 hr. after this dose the concentrations of bretylium in the tissues were considerably less, those in sympathetic ganglia being a half to one-quarter of those in the cats given 10 mg./kg. After 50 mg./kg. of bretylium all tissue levels at 18 hr. were roughly the same as those in cats given 10 mg./kg.

The possibility was considered that bretylium might penetrate into nerve axoplasm during the course of the ionic fluxes associated with impulse propagation and that this mode of entry would account for the rather slow rate of accumulation. A cat in which the right preganglionic cervical sympathetic nerve had been cut a month beforehand was given 10 mg./kg. labelled bretylium subcutaneously. Eighteen hours later the concentration of bretylium in the superior cervical ganglion of the sectioned side (122 mμmoles/g.) was similar to that of the intact side (117 mμmoles/g.) and there was no noteworthy difference in the amounts of bretylium in the postganglionic nerves (79 and 41 mμmoles/g. respectively). In an acute experiment, where the nerve of one side was cut and the other side left intact but stimulated supramaximally for 3 hr. after intravenous injection of the drug, there was again little difference between the bretylium contents of the superior cervical ganglia with their attached postganglionic nerves (cut, 232 mμmoles/g.; stimulated, 388 mμmoles/g.).

Topical Application.—Table III summarizes experiments in which various nerves were immersed in labelled bretylium, the degree of impairment of conduction assessed from the response of end organs, and the uptake of drug measured. The inferior cardiac and the hypogastric nerves took up the drug to concentrations exceeding those in the fluid in which they were immersed, within 20 to 100 min. Stimulation of the inferior cardiac nerve containing 600 mμmoles/g. no longer affected the heart rate, but, while conduction in the hypogastric nerve was apparently impaired when it contained an overall concentration of 90 mμmoles/g., blockade was still incomplete with 1,270 mμmoles/g. The preganglionic cervical

TABLE III

CONCENTRATION OF BRETYLIUM IN NERVES AFTER TOPICAL APPLICATION

The nerve, immersed in Tyrode solution containing [^{14}C]-labelled bretylium iodide, was stimulated at 15 pulses/sec. for 30 sec. every 3 or 5 min. Concentration of bretylium calculated from radioactivity determination.

	Response Tested	Bath Concentration of Bretylium Iodide mμmoles/ml.	Immersion Time in Min.	Inhibition of End Organ Response	Concentration of Drug in Nerve, mμmoles/g.
Adrenergic nerves					
Inferior cardiac	Heart rate	500	20	Complete	600
Hypogastric	Uterus	50	100	Partial	90
,,	,,	500	75	,,	1,270
Cholinergic nerves					
Preganglionic cervical sympathetic	Nictitating membrane	500	30	None	520
,,	,,	500	30	,,	500
,,	,,	1,000	30	,,	340
,,	,,	1,000	90	Partial	310
Greater splanchnic	Rise in B.P.	500	30	,,	90
,, ,,	,, ,,	500	35	,,	50
Phrenic	Diaphragm	500	30	None	90

sympathetic nerve also readily absorbed the drug, but an impairment of conduction was apparent in only 1 of 4 preparations. It may be significant that the nerve which showed blockade, though containing no greater concentration of the drug, had been immersed 90 min. instead of 30 min. The longer period of immersion might perhaps have allowed deeper penetration into the nerve. In an experiment not shown in the Table, the drug was taken up readily not only by the preganglionic cervical sympathetic trunk but also by the superior cervical ganglion and its postganglionic nerve trunk; these structures contained 850, 420, and 540 mμmoles/g. respectively after they had been immersed together for 30 min. in bretylium at a concentration of 500 mμmoles/ml. The response of the nictitating membrane to preganglionic stimulation was abolished.

The greater splanchnic and the phrenic nerve (Table III) took up bretylium to only about a fifth of the concentration of the solution in which they were immersed, within 30 min. Some impairment of the pressor response to stimulation of the splanchnic nerve containing 50 to 90 mμmoles/g. was apparent, but supramaximal stimulation of the phrenic nerve containing 90 mμmoles/g. continued to cause maximal contractions of the diaphragm.

Similar results were obtained with topical application of unlabelled bretylium bromide. The inferior cardiac nerve was blocked by immersion in a solution of 185 mμmoles/ml. for 13 min. (one experiment), and the postganglionic cervical sympathetic nerve was blocked by 450 mμmoles/ml. in 25 min. (one experiment). No impairment of conduction was apparent in preganglionic cervical sympathetic nerves immersed for 30 min. in bretylium at 1,000 to 2,700 mμmoles/ml. (five experiments).

Discussion

The following conclusions are based on the assumption that the radioactivity found in samples was accounted for solely by unaltered drug. Bretylium has been found to suffer negligible metabolic alteration in contact with tissues (Duncombe and McCoubrey, 1960).

The most interesting feature in the distribution of bretylium after subcutaneous injection in the cat is the slow accumulation of very high concentrations of the drug by sympathetic ganglia and their postganglionic nerve trunks. Moreover, the results for various time intervals show that the levels in these tissues are temporally related to the degree of relaxation of the nictitating membrane, a manifestation of the adrenergic neurone blocking action of the drug (Boura and Green, 1959). Other ganglia and nerve tissues never attained such high concentrations of the drug, and the specificity of the blocking action of bretylium on adrenergic nerves (Boura and Green, 1959) may therefore be related to the preferential accumulation of the drug by the adrenergic neurones. The sympathetic ganglia contained higher concentrations than did their postganglionic trunks, and this may be due to their having a relatively smaller proportion of supporting tissue.

Several of the major organs in the body accumulated tissue levels far exceeding those in the blood, but the concentrations attained were considerably less than those in adrenergic neurones and, except in the spleen, reached their peak and declined before the onset of sympathetic block. In experiments in guinea-pigs and rats (McCoubrey, unpublished), the spleen did not retain high concentrations of bretylium. In general, organs known to contain high concentrations of catechol amines accumulated the drug, but there is no clear relationship between their catechol amine concentrations and their degree of retention of the drug. In particular, the concentration in the adrenal medulla was far less than that in adrenergic neurones. At the highest concentration found in the superior cervical ganglion, there were about 10 molecules of bretylium present for every molecule of noradrenaline. It is interesting that, although bretylium did not penetrate into the cerebral cortex and spinal cord, small amounts were detected in the hypothalamus and area postrema, regions known to contain noradrenaline (Vogt, 1954).

Bretylium has a very persistent local anaesthetic action and blocks conduction in the adrenergic nerve trunks supplying the rabbit intestine, uterus and ear vessels when topically applied in a concentration of 37 to 370 mμmoles/ml. (Boura and Green, 1959). The present experiments show that with topical application block occurs in the postganglionic (adrenergic) nerves of the cat when they contain an overall concentration similar to that found when blockade has been produced by a subcutaneous dose. However, the distributions within the nerve cannot be expected to be the same in the two cases. Nerves of other types could also be blocked by topically applied drug but, in general, those examined were less sensitive. The available evidence is not sufficient to show how far the degree of block is related to the thickness of nerve trunks or to the ease of access to individual fibres. The differences observed after topical application of the drug, for example the high sensitivity of postganglionic fibres and the

insensitivity of the phrenic nerve, are compatible with myelin acting as a barrier to the bretylium ion. Fibre size may also be important in relation to sensitivity, as it is with other local anaesthetics. These factors are presumably of importance not only when the drug is applied topically but also when it is given systemically.

We conclude that bretylium may act in a manner analogous to that of the local anaesthetic drugs, as was originally postulated to explain the adrenergic nerve blocking action of choline 2,6-xylyl ether bromide (Hey and Willey, 1954). Its specific action on adrenergic neurones may be related to their ability to selectively accumulate the drug, as well as to the greater sensitivity of finer and less protected fibres. It seems reasonable to assume that the whole of the adrenergic neurone is susceptible to bretylium, but that the ease of access and therefore of block will be influenced by differences in the richness of the local blood supply. The vascularity of the ganglia appears to be greater than that of nerve trunks, and this is in keeping with their more rapid attainment of a blocking concentration. The drug may also have a comparatively ready access to the nerve terminals, and after subcutaneous injection these may well suffer impairment of function first.

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THE RELATIONSHIP BETWEEN THE CHEMICAL STRUCTURE AND NEUROTOXICITY OF ALKYL ORGANOPHOSPHORUS COMPOUNDS

BY

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Thirty-six alkyl organophosphorus compounds have been tested for neurotoxicity in the chicken. The individual compounds were chosen to enable the importance of each portion of the molecule to be assessed in relation to the property of neurotoxicity. Seventeen substances were found to be neurotoxic, fifteen for the first time. All of these contained fluorine. On the basis of the results reported, certain predictions have been made about the chemical structure of compounds which would be expected to be neurotoxic. The importance of fluorine suggests that it plays a direct role in the development of the biochemical lesion, and this may occur as the result of its being carried by the molecule as a whole to specific areas in the nervous system. By the action of cholinesterase, the P–F bond may be ruptured and ionic fluorine liberated where it blocks some metabolic cycle.

Ginger paralysis, Jake paralysis, Jamaica ginger polyneuritis or triorthocresyl phosphate poisoning were some of the names given to a condition which afflicted a very large number of people throughout the Southern States of the United States of America during 1930 (Kidd and Langworthy, 1933). It was caused by the ingestion of an alcoholic extract of Jamaica ginger adulterated with tri-*o*-cresyl phosphate (Smith, Elvove and Frazier, 1930).

Since a similar condition has been observed in man after poisoning by *NN'*-diisopropylphosphorodiamidic fluoride (mipafox) (Bidstrup and Bonnell, 1954) and in chickens following diisopropyl phosphorofluoridate (dyflos), mipafox (Barnes and Denz, 1953) and a number of aryl phosphates other than tri-*o*-cresyl phosphate (Hine, Dunlap, Rice, Coursey, Gross and Anderson, 1956; Henschler, 1958), the names above are no longer satisfactory for this condition. Because the histological lesions produced by these substances are confined to the nervous system (Smith and Lillie, 1931; Barnes and Denz, 1953; Fenton, 1955; Cavanagh, 1954) it is proposed to refer to this condition as organophosphorus neurotoxicity or, for brevity, neurotoxicity.

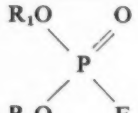
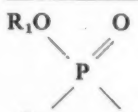
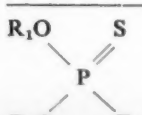
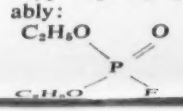
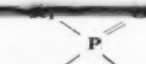
Detailed accounts of both the clinical and histological picture have been given by many writers, and the following due to Kidd and Langworthy (1933) is typical of the clinical picture in man after poisoning by tri-*o*-cresyl phosphate. Following a symptomless period of 5 to 30 days there occurs a tingling numbness or cramping pain in the calves, which lasts for 1 or 2 days. This is followed by a rapid onset of complete or partial paralysis of the extensor muscles of the toes and feet, together with variable degrees of weakness, but always with loss of ankle jerks. A week later the muscles of the hand and forearm develop a sudden weakness and bilateral paralysis involving both sides to an equal extent. The course of the illness is chronic and little improvement can be observed for months after onset. Even when it occurs, progress is slow.

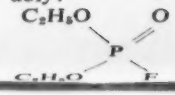
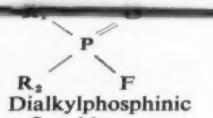
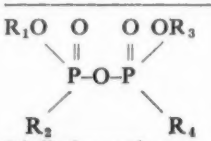
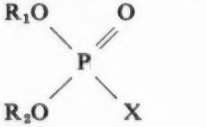
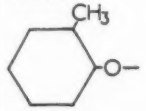
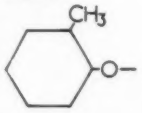
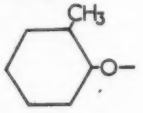
There is considerable variation in both the clinical and histological appearances of the disease in various species (Smith, Elvove and Frazier, 1930) and it is only in the hen and the cat that the appearances closely resemble those seen in man (Smith, Engel and Stohlman, 1932). Most experimental work has been carried out in chickens where the histological lesions are limited to the ascending and descending fibres of the sciatic nerve, the spinal cord and the cerebellum. The changes consist of myelin and axon degeneration (Cavanagh, 1954; Fenton, 1955).

Previous to the work to be described in this paper only a limited number of compounds were

TABLE I

CHEMICAL CONSTITUTION AND PHYSICAL CONSTANTS OF ALL THE COMPOUNDS EXAMINED

Type of Compound	R_1	R_2	R_3	R_4	X	b.p./mm.Hg	Refractive Index n_D^{25} Found	Remarks
 Phosphorofluoridates	CH_3–	CH_3–				46°/12	1·3553	
	C_2H_5–	C_2H_5–				59°/10	1·3708	
	C_3H_7–	C_3H_7–				54°/2·0	1·3875	100% pure by Schoneman reaction
	i-C_3H_7–	*i*-C_3H_7–				40°/1·0	1·3795	
	C_4H_9–	C_4H_9–				68°/0·2	1·3993	C. & H. analysis. Found C. 45·44%. H. 8·39%. Calculated C. 45·28%. H. 8·55%
	i-C_4H_9–	*i*-C_4H_9–				50°/0·5	1·3955	
	s-C_4H_9–	s-C_4H_9–				59°/1·0	1·3935	
	C_5H_{11}–	C_5H_{11}–				68°/0·1	1·4080	C. & H. analysis. Found C. 50·11%. H. 9·30%. Calculated C. 49·99%. H. 9·23%
	$C_3H_7.CH(CH_3)$–	$C_3H_7.CH(CH_3)$–				63°/0·2	1·4119	
	Cyclohexyl–	Cyclohexyl–				82°/0·01	1·4552	C. & H. analysis. Found C. 54·44%. H. 8·56%. Calculated C. 54·53%. H. 8·39%
	C_2H_5–	C_3H_7–				71°/10·0	1·3787	
 Alkylphosphonofluoridates	*i*-C_3H_7–	CH_3–						
	i-C_3H_7–	C_2H_5–						
	CH_3–	*i*-C_3H_7–				55°/13	1·3869	
	C_2H_5–	CH_3–				64°/23	1·3810	
	i-C_4H_9–	CH_3–				46°/1·5	1·3922	
 Phosphorofluoridothionate	C_2H_5–	C_2H_5–				68°/24	1·4208	Infra-red spectrum shows the presence of approx. 10% impurity probably: 
	C_3H_7–	C_3H_7–				76°/1·5	1·4185	
	i-C_3H_7–	*i*-C_3H_7–				52°/0·5	1·4220	
						74°/0·2	1·4275	

Phosphorofluoridothionate								
 Dialkylphosphinic fluorides	C_2H_5– C_3H_7– *i*-C_3H_7– C_4H_9–	C_2H_5– C_3H_7– *i*-C_3H_7– C_4H_9–				48°/0·5 76°/1·5 52°/0·5 74°/0·2	1·4123 1·4185 1·4220 1·4275	
 Dialkylpyrophosphonates	C_2H_5– C_2H_5– C_4H_9– C_2H_5– *i*-C_3H_7– C_2H_5–	C_2H_5O– CH_3– CH_3– CH_3– CH_3– CH_3–	C_2H_5– C_3H_7– C_4H_9– C_2H_5– *i*-C_3H_7– C_2H_5–	C_2H_5O– CH_3– CH_3– C_2H_5– CH_3– CH_3–			1·4166 1·4348 1·4360 1·4350 1·4298 1·4348	Ethyl pyrophosphate
 " Miscellaneous " compounds	C_2H_5– C_2H_5–	C_2H_5– C_2H_5–			Cl– CN–	47°/1·5 43°/0·1	1·4155 1·4009	Nitrogen determination. Found 8·51%. Calculated 8·59%
	C_2H_5– C_3H_7–	C_2H_5– C_3H_7–			C_2H_5O– 	102°/0·035	1·4770	C. & H. determination. Found C. 56·33%. H. 7·78%. Calculated C. 57·34%. H. 7·77%
	i-C_3H_7– C_4H_9– *i*-C_4H_9– C_2H_5–	*i*-C_3H_7– C_4H_9– *i*-C_4H_9– C_2H_5O–			$C_6H_5.NH$– Cl– Cl– $(C_2H_5)_2N.[CH_2]_2.S$–	120° (m.p.) 78°/0·2 76°/0·7	 1·4294 1·4254	 Refractive index n_D^{15} Oxalate used—solid
					CH_3–	130°/0·05	1·5335	

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known to be neurotoxic. These were dyflos, mipafox and certain triaryl phosphates. Consequently it was not possible to recognize any correlation between chemical structure and neurotoxicity.

This paper presents the results of a systematic study of the relationship between the structure of certain organophosphorus compounds and neurotoxicity. A number of these compounds have been shown for the first time to be neurotoxic. All of these contain fluorine ; in addition the significance of the other groups in the molecule has been worked out.

Methods

Chemical Methods

Phosphorofluoridates.—The majority of these were prepared by the method of Ford-Moore, Lermit and Stratford (1953). This was modified for the preparation ethyl propyl phosphorofluoridate (British Intelligence Objectives Subcommittee, 1945) and for di(1-methylbutyl) phosphorofluoridate (Goldwhite and Saunders, 1955).

Diethyl Phosphorofluoridothionate. — This was prepared from the phosphorochloridothionate, replacing the chlorine atom by a fluorine atom, by the action of antimony trifluoride in the presence of antimony pentachloride.

Alkylphosphonofluoridates.—These were obtained by the action of phosgene on the appropriate alkyl alkylphosphonate to give the alkylphosphonochloridate, and this on treatment with sodium fluoride gave the alkylphosphonofluoridate (Coe, Perry and Brown, 1957).

Dialkylphosphinic Fluorides.—Treatment of the corresponding chlorides with a mixture of sodium fluoride and sodium bifluoride gave the dialkylphosphinic fluorides.

Dialkylpyrophosphonates.—These were obtained pure by reacting the silver salt of an alkyl hydrogen alkylphosphonate with an alkyl alkylphosphonochloridate (Kosolapoff, 1950).

Tests of Purity and Identification.—The purity and identity of all the compounds were established by examination of the infra-red spectrum, determination of the refractive index and, in some cases, determination of the carbon and hydrogen. With one exception, which is discussed in the text, all the compounds were at least 95% pure at the time of testing. The chemical structure and physical constants of the 36 compounds examined are shown in Table I.

Biological Methods

Chickens of mixed breed and both sexes were used throughout, and since age is significant in the development of neurotoxic effects all the birds used were at least 18 months old (Barnes and Denz, 1953).

The agents were given intramuscularly as a 1% dilution in 10% ethanol saline (Austin and Davies, 1954), within 0.5 hr. of preparation of the solution. Where an agent was known to be unstable or insoluble in water the solution was made up in isopropanol. Slight deviations from this procedure were made when the dose to be administered was greater than 10 mg./kg. Under these circumstances the concentration of the agent was adjusted to keep the volume of the injected solution between 0.2 and 1.0 ml.

Many of the agents used were so lethal that each bird had to be protected by the intramuscular injection of 2-hydroxyiminomethyl-*N*-methylpyridinium methanesulphonate (P2S) 100 mg./kg. and of atropine sulphate 1 mg./kg. 10 min. before the injection of the agent. Preliminary experiments with dyflos showed that, whilst such treatment saved the lives of birds given many times a lethal dose, it had no effect upon either the incidence, the severity or the onset of neurotoxic effects. Despite the protective effect of P2S and atropine some compounds were so toxic that the dose levels necessary for adequate testing could only be given in 20% daily aliquots of the total.

Occasionally, for very specific reasons, P2S was not administered. In these instances the dose of atropine was increased and this is indicated either in the tables or the text.

In the chicken the interval between poisoning and the onset of paralysis is always between 9 and 14 days ; all birds were therefore observed for a period of 21 days from the completion of the injections. The absence of bilateral leg weakness and ataxia at the end of this period was taken as a negative result and the compound was considered inactive. With a number of the compounds, particularly those which did not produce leg weakness or ataxia, the period of observation was longer. The onset of such signs in a shorter interval than 7 days was regarded as atypical and the test was repeated.

All the birds were killed either at the end of the observation period or when they developed unequivocal clinical signs. The sciatic nerves, spinal cord and cerebellum of many of these clinically positive birds were examined histologically.

Results

The Acute Effects of Organophosphorus Compounds in the Presence of P2S and Atropine

With the exception of four compounds, all the agents were tested in the presence of P2S and atropine, with the result that very much larger doses of agent could be administered than was otherwise possible. For example, the LD50 of sarin injected intramuscularly to hens is approximately 50 μg./kg. whereas 20 times this dose was given in the presence of P2S and atropine without lethal effects.

Despite protection against the lethal action of these substances acute signs of anticholinesterase poisoning invariably occurred, and at the highest

dose levels some birds died. The effects varied in severity with the class of compound and with the individual members of any class. Immediate collapse followed by violent convulsions was a characteristic feature of poisoning by doses of 0.5 mg./kg. or more of both phosphonic and phosphinic fluorides. The protective effect of P2S and atropine was more marked in the case of the phosphorofluoridates. Although the rate of recovery was more rapid after this class of compound it was mainly dependent upon the actual substance.

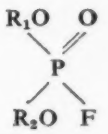
Large doses of dialkylpyrophosphonates could be given after P2S and atropine, without causing much distress. Although collapse and convulsions occurred, these were much less frequent and much less severe. For example, after the administration of ethyl pyrophosphate 50 mg./kg., only slight signs were observed and recovery was complete within 1 hr.

The Neurotoxicity of Organophosphorus Compounds

The Phosphorofluoridates.—Eleven compounds of this type were examined (Table II). All of them, with the exception of dimethyl phosphorofluoridate, were neurotoxic at dose levels between 0.3 mg. to 2.5 mg./kg. The dimethyl compound was neurotoxic at 30 mg./kg., but this may reflect the hydrolytic instability of this compound. In general, however, all the phosphorofluoridates were neurotoxic at about the same level, and neither the character of the alkyl groups nor the fact that they may be straight or branched chains seemed to influence this neurotoxic property. The onset of ataxia was markedly uniform, first signs

TABLE II

THE NEUROTOXICITY OF PHOSPHOROFLUORIDATES IN CHICKENS

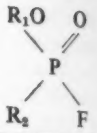


Each chicken was injected intramuscularly with P2S 100 mg./kg. and atropine sulphate 1 mg./kg., 10 min. before intramuscular injection of the agent.

R_1	R_2	Range of Doses Tested (mg./kg.)	Maximum Dose at which No Hens were Ataxic (mg./kg.)	Minimum Dose at which Ataxia was Observed (mg./kg.)	Proportion of Birds Positive at Minimum Dose or Greater	Mean Time of Onset of Ataxia (Days)	Remarks
CH_3–	CH_3–	1·5–40	20	30	1/1	11	
C_2H_5–	C_2H_5–	0·25–5·0	0·5	0·75	26/27	10	1 bird died from acute effects
C_3H_7–	C_3H_7–	0·1–1·0	0·1	0·25	12/13	12	,, ,,
i-C_3H_7–	*i*-C_3H_7–	0·2–1·0	0·2	0·3	10/12	10	2 birds showed no signs at 0·3 mg./kg.
C_4H_9–	C_4H_9–	0·5–5·0	—	0·5	12/14	9	1 bird showed no signs. 1 bird died from acute effects
i-C_4H_9–	*i*-C_4H_9–	1·5–5·0	—	1·5	6/6	14	
s-C_4H_9–	s-C_4H_9–	1·5–5·0	—	1·5	5/6	12	1 bird died from acute effects
C_5H_{11}–	C_5H_{11}–	1·0–40·0	1·0	2·5	7/11	10	4 birds showed no signs
$C_3H_7.CH(CH_3)$–	$C_3H_7.CH(CH_3)$–	2·5–10·0	—	2·5	4/6	9	2 birds died from acute effects
cyclohexyl-	cyclohexyl-	1·0–5·0	1·0	2·5	3/4	10	1 bird died from injury
C_3H_7–	C_3H_7–	1·0–5·0	—	1·0	5/6	12	1 bird died from acute effects

TABLE III

THE NEUROTOXICITY OF ALKYLPHOSPHONOFLUORIDATES IN CHICKENS



Dose schedule as described in Table II.

R_1	R_2	Range of Doses Tested (mg./kg.)	Maximum Dose at which No Hens were Ataxic (mg./kg.)	Minimum Dose at which Ataxia was Observed (mg./kg.)	Proportion of Birds Positive at Minimum Dose or Greater	Mean Time of Onset of Ataxia (Days)	Remarks
i-C_3H_7–	CH_3–	0·5–2·5	0·5	1·0	9/28	12	In general the total dose was built up by giving 20% daily aliquots. 19 birds died during period of dosing
i-C_3H_7–	C_2H_5–	1·0–5·0	—	1·0	5/6	13	1 bird died of acute effects
CH_3–	*i*–C_3H_7–	2·5–15·0	2·5	5·0	3/8	10	2 birds died of acute effects at 15 mg./kg. 3 birds showed no signs
C_2H_5–	CH_3–	1·0–2·9	2·25	2·9	5/7	11	2 birds showed no signs
i-C_4H_9–	C_2H_5–	2·9	—	2·9	2/6	6	4 birds died from acute effects. Dosing was spread over 7 days. The delay period was therefore difficult to assess

always being noticeable 9 to 15 days after poisoning (Table II).

The Alkylphosphonofluoridates.—The toxicity of the alkylphosphonofluoridates was so great that demonstration of their neurotoxicity was very difficult. Treatment with P2S and atropine was consistently effective in protecting birds against the lethal effects of 0.5 mg./kg., but not against higher doses. These were therefore given in divided doses, usually 20% daily aliquots of the total dose to be tested. Where this was done is indicated in Table III. Five compounds were examined at doses ranging from 0.5 to 15.0 mg./kg. All five were active between 1.0 and 5.0 mg./kg. (Table III).

The Phosphorofluoridothionates.—Diethyl phosphorofluoridothionate was examined in order to determine the significance of the oxygen atom directly attached to the phosphorus atom. It was neurotoxic at 5.0 mg./kg., but the sample contained 10% of an impurity, which was probably the oxygen analogue.

Diethyl phosphorofluoridate was therefore re-tested under comparable conditions. It was non-neurotoxic at 0.5 mg./kg. and neurotoxic at 0.75 mg./kg. Thus even if all the impurity present in the sample of diethyl phosphorofluoridothionate was the oxygen analogue it would not be sufficient to account for the neurotoxicity of the sulphur compound.

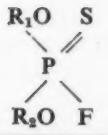
The Dialkylphosphinic Fluorides.—Four compounds of this type were tested. Their formulae are shown in Table I. In a preliminary experiment the di-*n*-propyl and the di-*n*-butyl analogues were examined, in the presence of P2S and atropine at maximum doses of 5.0 and 2.5 mg./kg. respectively. Neither was neurotoxic. Since it was thought that the oxime might catalyse the breakdown of the agent, all four compounds were tested in the absence of the P2S, but the dose of atropine was increased to 10 mg./kg. Again the compounds produced no effect, although one bird given 5.0 mg./kg. di-*n*-propylphosphinic fluoride exhibited atypical symptoms 27 days after poisoning.

The Dialkylpyrophosphonates.—Five dialkyl dialkylpyrophosphonates and ethyl pyrophosphate were examined. With the exception of ethyl pyrophosphate they were tested at doses varying from 1 to 10 mg./kg. Ethyl pyrophosphate was tested more vigorously, cumulative doses of up to 100 mg./kg. being administered over a period of a week. The period of observation was also prolonged and in some cases lasted for 56 days. None of these compounds produced neurotoxicity. It is possible that the maximum doses of 10 mg./

TABLE IV

THE NEUROTOXICITY OF PHOSPHOROFLUORIDOTHIONATES

Dose schedule as described in Table II.



R_1	R_2	Range of Doses Tested (mg./kg.)	Maximum Dose at which No Hens were Ataxic (mg./kg.)	Minimum Dose at which Ataxia was Observed (mg./kg.)	Proportion of Birds Ataxic at Minimum Dose or Greater	Mean Time of Onset of Ataxia (Days)	Remarks
C_2H_5–	C_2H_5–	2·5–7·5	2·5	5·0	4/4	7	Sample contained 10% impurity—(infra-red)

kg. were too low to define adequately a negative response. The intrinsic toxicity of these compounds, however, is so high that even with these comparatively small quantities the birds received many times the LD50 dose.

Miscellaneous Compounds.—The formulae of nine miscellaneous compounds investigated are also shown in Table I. These are essentially "phosphorofluoridates" in which the fluorine has been replaced by other radicals. These were also non-neurotoxic, even though some were tested at 50 to 100 mg./kg., for example, diethyl phosphorocyanidate, diisopropyl phosphoramidate, di-*o*-cresyl methyl phosphate and diethyl phosphorochloridate.

DISCUSSION

Thirty-six organophosphorus compounds have been examined for neurotoxicity. The various classes of compounds and the individual members of each were chosen with the object of assessing the importance which the individual groups, attached to the phosphorus atom, have in producing these effects. The results are summarized in Table V.

All the phosphorofluoridates tested were neurotoxic, from the dimethyl to the di(1-methylbutyl) homologue, suggesting that the actual nature of the ester alkyl group is not critical; this is supported by the observation that dicyclohexyl phosphorofluoridate and the mixed alkyl ester, ethyl propyl phosphorofluoridate, also produce neurotoxicity.

At least one alkoxyl group seems to be necessary, for the alkylphosphonofluoridates are neurotoxic, whereas the dialkylphosphinic fluorides are not. The ester oxygen of the phosphorofluoridates may be replaced by an amino-group as in mipafox, but

TABLE V

NEUROTOXICITY OF VARIOUS CLASSES OF ORGANOPHOSPHORUS COMPOUNDS

Class of Compound	No. of Compounds Tested	No. Positive
Phosphorofluoridates ..	11	11
Alkylphosphonofluoridates	5	5
Phosphorofluoridothionate	1	1
Dialkylphosphinic fluorides	4	0
Dialkylpyrophosphonates and ethyl pyrophosphate ..	6	0
Miscellaneous compounds ..	9	0

it is not known whether an alkyl *N*-alkylphosphoramidofluoridate would be neurotoxic. The phosphoryl oxygen may be replaceable by sulphur, but, in view of the possible presence of the oxygen analogue of diethyl phosphorofluoridothionate as an impurity in the only compound of this type examined, this is by no means established.

The most significant structure is the fluorine atom, for replacement of this atom with a wide variety of groups always results in loss of neurotoxicity. Thus a compound with the general formula, I, will possess neurotoxic properties, provided that R_1 is an alkyl group, R_2 is an alkyl, alkoxyl or alkylamino-group, A is an oxygen or secondary amino-group, and B is an oxygen or possibly a sulphur atom.

(I)

The significance of the fluorine atom suggests that it plays a direct role in the development of the biochemical lesion. This may result from its being carried by the compound as a whole to specific areas in the nervous system. At these sites ionic fluorine may be liberated by rupture of the P-F bond. This may occur by the action of cholinesterase, but this need not be the only mechanism involved. The need to deposit the fluorine at these specific sites determines the character of the rest of the molecule.

Substitution of the fluorine atom by a chlorine or bromine atom results in a very unstable molecule which almost certainly does not penetrate the central nervous system intact. This is borne out by the lack of toxicity of such compounds. Compounds like the dialkyl dialkylpyrophosphonates are more stable, and there is little doubt as judged by their acute toxic effects that they enter the central nervous system. An explanation for their lack of neurotoxicity must therefore be sought elsewhere. The hypothesis offered above is capable, however, of explaining the absence of neurotoxicity of these substances, for the rupture of the P-X bond in different compounds will result in the production *in vivo* of a variety of substances with markedly different properties. Thus the pyrophosphates would yield derivatives of orthophosphoric acid, and these do not possess properties similar to hydrofluoric acid.

Whilst this working hypothesis offers at least a partial explanation of the neurotoxicity of organophosphorus compounds of type I, it must not be forgotten that triaryl phosphates are similarly neurotoxic. This hypothesis, however, can be modified to cover such compounds, but its adaptation to these cases will be considered in a later communication.

The neurotoxic hazard which these substances constitute to man is a difficult problem to assess because of (*a*) known species differences in the response to organophosphorus compounds, and (*b*) the absence of any direct evidence that organophosphorus compounds other than tri-*o*-cresyl phosphate and mipafox are neurotoxic in humans. Both of these have been extensively examined in the chicken. The delay period, the clinical character of the condition and the distribution and nature of the histological lesions are all the same as those found with dyflos and the compounds which have, in this study, been reported active for the first time. It must therefore be assumed that all the organophosphorus compounds shown to be neurotoxic in chickens will, under appropriate conditions, produce neurotoxicity in man.

The authors wish to acknowledge the help given by Mr. W. Pearce with the animal experiments.

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A NOTE ON THE DEMYELINATION PRODUCED IN HENS BY DIALKYLFLUORIDATES

BY

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From the Microbiological Research Establishment, Porton, Salisbury, Wilts

(RECEIVED DECEMBER 22, 1959)

Hens were treated with organophosphorus compounds until signs of neurotoxicity developed. The sciatic nerves, spinal cord, and medulla were then examined histologically by the Marchi method, to determine the extent and distribution of demyelination. The lesions were proportional to the dose of compound injected, and the spinal cord was more sensitive than the sciatic nerves. It is suggested that the spinal cord, and not the sciatic nerves, is the main site of damage in clinical paralysis following dyflos poisoning.

In order to confirm the clinical findings described in the previous paper (Davies, Holland, and Rumens, 1960), a number of active compounds were examined for their ability to produce demyelination in the hen. The distribution of lesions in the peripheral and central nervous system following organophosphorus administration has been described by Barnes and Denz (1953), Fenton (1955), and Cavanagh (1954), who found demyelination in the sciatic nerve, spinal cord, medulla, and cerebellum.

METHODS

The sciatic nerves, spinal cord, and medulla or cerebellum from seriously ataxic or paralysed birds were taken for histological examination. The birds were killed by the injection of pentobarbitone, perfused, and tissues taken as described by Barnes and Denz (1953). They were stained by the modified Marchi staining methods of Swank and Davenport (1935), Poirier, Ayotte and Gauthier (1954), and Adams (1958). Using these methods the degenerating myelin stains black, and, for recording, all the positive sections were scored from + to +++ depending upon the intensity of the staining. This method is used in Table I in which are summarized the effects of nine compounds to indicate the severity of the lesions. All sections were made at comparable levels in each tissue.

Normal controls and diisopropyl phosphorofluoridate(dyflos)-poisoned birds were similarly treated for comparison with other compounds.

RESULTS

Although all these compounds produced demyelination in the spinal cord, some failed to do so in the sciatic nerve, but there was no essential difference in the distribution of lesions

TABLE I

DEMYELINATION OF THE SCIATIC NERVE, SPINAL CORD AND MEDULLA IN HENS AFTER POISONING WITH CERTAIN ORGANOPHOSPHORUS COMPOUNDS

All comparisons of Osmium staining were made at similar levels in corresponding tissues.

	Dose (mg./kg.)	Clinical Effects	Demyelination		
			Sciatic Nerve	Spinal Cord	Medulla
Phosphorofluoridates					
1. Diisopropyl	0·2	None	−	−	−
	0·4	Slight	−	+	−
	0·6	Definite	−	++	−
	0·9	Severe	+	++	+
2. Diethyl ..	2·5	,,	+	++	+
3. Dibutyl ..	2·5	,,	++	++	+
4. Dipentyl	20·0	,,	++	++	+
Phosphorofluoridothionate					
5. Diethyl ..	7·5		++	++	−
Alkylphosphonofluoridates					
6. Isopropyl ethyl-	1·0	Slight	−	+	−
	5·0	Severe	+	+++	++
7. Ethyl methyl-	2·9	,,	+	+++	+
8. Isobutyl methyl-	2·9	,,	+	++	−
9. Methyl isopropyl-	5·0	,,	+	+++	+

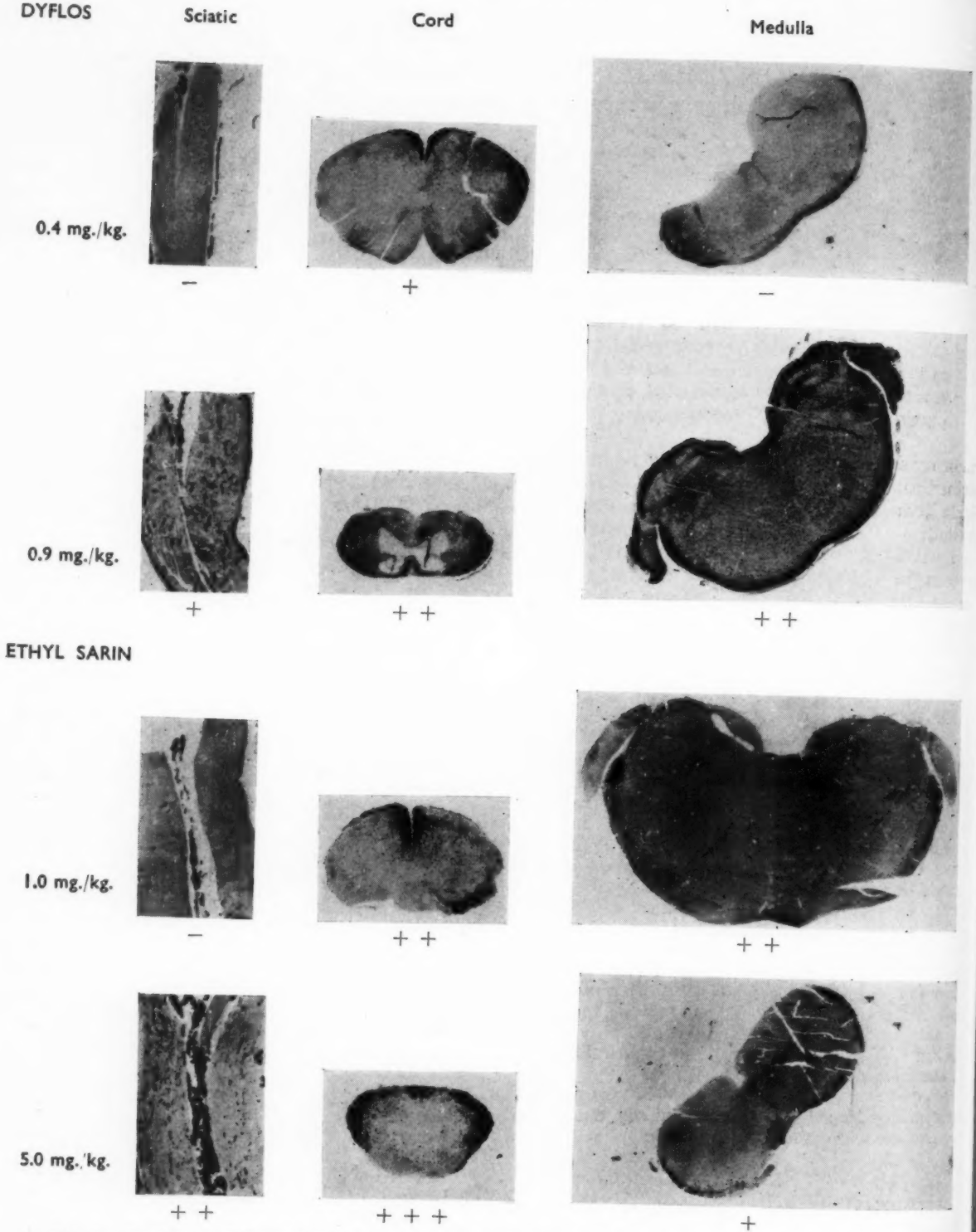

FIG. 1.—Sections at different levels of sciatic nerve, spinal cord and medulla illustrating demyelination in hens poisoned with dyflos and ethyl sarin (magnification × 8).

from that produced by dyflos. The distribution and severity of lesions produced by dyflos are summarized in Table I. The effects are proportional to the dose over the range 0.2 mg./kg. to 0.9 mg./kg. Their extent and distribution are similar to that described by Fenton (1955). It should be noted that the low dose levels produced lesions in the spinal cord but not in the sciatic nerve, and this clearly demonstrates the greater sensitivity of the spinal cord to damage by the fluoridates.

For comparison the distributions of Marchi staining after dyflos 0.4 mg./kg. and 0.9 mg./kg. and isopropylethylphosphonofluoridate (ethyl sarin) 1.0 mg./kg. and 5.0 mg./kg. are shown in Fig. 1, and illustrate the comparable distribution of demyelination obtained with both these compounds.

The importance of the sciatic nerve lesions in the clinical paralysis with dyflos seems to have been over-emphasized, for the present results indicate that it is the spinal cord which is the main site of damage.

The distribution of the histological lesions produced by the other fluoridates is identical to that seen after dyflos poisoning, and since the clinical picture is also closely similar to that caused by these other compounds, it is highly probable that all the fluoridates produce an identical clinical and pathological neurotoxic syndrome.

The author wishes to thank Miss G. Meaden for preparing the Marchi sections, Mr. G. B. Carter for the Adams method, and Mr. E. Cuff for the photomicrographs.

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THE INFLUENCE OF THIOPENTONE ANAESTHESIA ON THE BLOOD LIPID AND BLOOD SUGAR LEVEL

BY

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From the Institute for Cardiovascular Research, Prague, Czechoslovakia

(RECEIVED JANUARY 1, 1960)

Thiopentone anaesthesia in dogs and rats was accompanied by a sharp fall in blood non-esterified fatty acids and a small increase in blood sugar. No pronounced changes in the blood concentrations of cholesterol, phospholipids or fatty acid esters were observed. Ether anaesthesia had no effect on the blood non-esterified fatty acids in rats. The fall in non-esterified fatty acids during thiopentone anaesthesia is therefore not related to the state of anaesthesia itself.

Thiopentone has a special position among anaesthetics of the barbiturate series. It acts almost immediately and its action is of very short duration. This latter property of thiopentone is due to its ready solubility in body fat (Brodie, Bernstein, and Mark, 1952), and the amount of body fat is the main factor influencing the duration of thiopentone anaesthesia. A decrease in body fat prolongs the duration of anaesthesia (Hermann and Wood, 1952). On the other hand, alimentary lipaemia in rats reduces the duration of anaesthesia due to a partial binding of thiopentone by blood lipids (Anderson and Magee, 1953).

In view of the great affinity of thiopentone for body fat, the possibility that other metabolic changes, particularly in lipid metabolism, might occur during thiopentone anaesthesia has been investigated. Valuable information in this respect can be obtained from investigations of the level of non-esterified fatty acids, an important lipid fraction in the mobilization, transport and utilization of fats.

METHODS

The experiments were carried out on dogs of both sexes, fasted for 12 hr. prior to experiments, and on 7-month-old albino rats kept on a standard laboratory

TABLE I

CONCENTRATIONS (M.EQUIV./L.) OF NON-ESTERIFIED FATTY ACIDS IN THE BLOOD OF CONTROL DOGS (1–5) AND OF DOGS (6–10) ANAESTHETIZED WITH THIOPENTONE

The initial sample (0) was taken just before the injection. Subsequent samples were taken at the times indicated up to 2 hr.

Treatment	Dog No.	Time in min.								
		0	10	30	40	60	70	90	100	120
Intravenous saline	1	0·37	—	0·37	—	0·42	—	0·42	—	0·48
	2	0·58	0·53	—	0·74	—	0·79	—	0·69	0·93
	3	0·89	0·85	—	—	0·90	—	0·89	—	0·93
	4	1·01	0·98	—	1·03	—	0·98	—	1·03	0·98
	5	0·74	0·90	0·85	—	0·85	—	0·90	—	0·95
Intravenous thiopentone	6	0·37	0·37	0·21	—	0·19	—	—	—	0·16
	7	0·77	0·74	0·58	—	0·48	—	0·42	—	0·37
	8	0·66	0·45	—	0·40	—	0·40	—	0·34	—
	9	0·62	0·53	—	0·37	—	0·37	—	0·32	—
	10	0·69	—	0·48	0·42	—	—	—	—	—

TABLE II

MEAN CONCENTRATIONS (±S.E.) OF LIPID FRACTIONS AND GLUCOSE IN THE SERUM OF 5 DOGS DURING THIOPENTONE ANAESTHESIA

Time (min.)	Non-esterified Fatty Acids m.equiv./l.	Total Cholesterol mg./100 ml.	Phospholipids mg./100 ml.	Fatty Acid Esters mg./100 ml.	Blood Sugar mg./100 ml.
0	1·36 ± 0·34	133 ± 44	363 ± 87	335 ± 68	70 ± 15
30	0·85 ± 0·18	125 ± 40	339 ± 85	306 ± 63	90 ± 8
60	0·81 ± 0·19	125 ± 44	349 ± 93	307 ± 74	102 ± 17
90	0·82 ± 0·27	125 ± 44	347 ± 81	310 ± 69	102 ± 8
120	0·69 ± 0·40	125 ± 46	348 ± 88	305 ± 77	104 ± 9

diet. Anaesthesia was induced by injection of Thiopental-Spofa, a Czechoslovak preparation of thiopentone.

Series 1.—Thiopentone was administered intravenously to 5 dogs. Each dog received first an initial dose of about 30 mg./kg. thiopentone as a 2.5% solution in saline; later, supplementary injections were given to maintain anaesthesia. Each dog received a total of about 60 mg./kg. An equivalent amount of saline was administered to 5 control dogs. Venous blood was drawn from a limb and 1 ml. portions were immediately pipetted into an extraction mixture and the non-esterified fatty acids were estimated by Dole's method (Dole, 1956), using an alcoholic solution of Nile blue as indicator. At first, whole blood was employed to avoid spontaneous lipolysis which might occur during the separation of serum.

In a further group of 5 dogs blood was allowed to coagulate, since spontaneous lipolysis was found to be negligible under these conditions. In the corresponding serum samples, total cholesterol was estimated according to Abell, Levy, Brodie, and Kendall (1952), phospholipids by King's method (King, 1951) and fatty acids by the method of Stern and Shapiro (1953). Blood sugar was estimated by a ferricyanide-titrimetric method.

Series 2.—Six female rats were injected intraperitoneally with thiopentone 40 mg./kg. as a 2.5% solution in saline. The control animals received saline only. The animals were killed by decapitation 60 min. after thiopentone and the blood was collected into test tubes. After coagulation and centrifuging, sugar and non-esterified fatty acid were estimated in the serum.

Series 3.—Fourteen experimental rats were kept for 1 hr. under ether anaesthesia. The control group consisted of 16 untreated rats. Subsequent procedures were similar to those of series 2.

RESULTS

Series 1

The results of this series of experiments are summarized in Table I. It is evident that thiopentone anaesthesia caused a marked fall of non-esterified fatty acid concentrations in all the dogs. In the 5 control dogs the concentration showed a slight tendency to increase.

In another 5 dogs (Table II) the serum lipid and sugar concentrations during thiopentone anaesthesia were investigated. A marked decline of non-esterified fatty acid was observed, whereas the blood sugar values increased. These changes were greatest during the first hour of anaesthesia. Total cholesterol and phospholipid concentrations

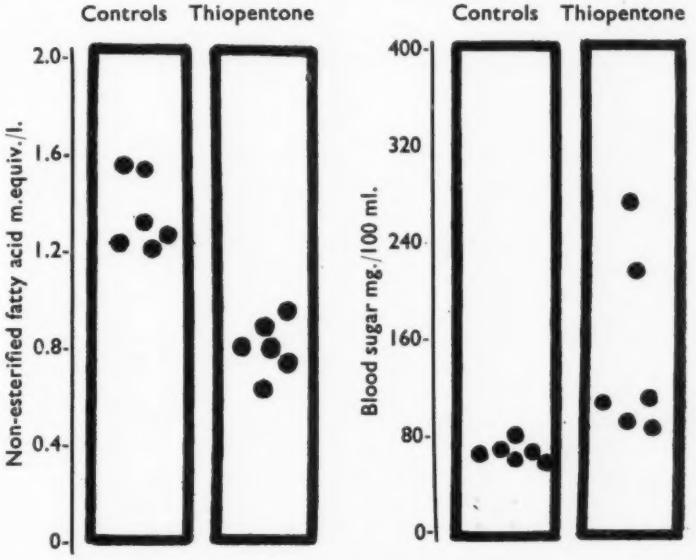

FIG. 1.—The effect of thiopentone on the concentrations of non-esterified fatty acids and sugar in rat blood. Six rats were injected intraperitoneally with thiopentone 40 mg./kg. and six control rats with saline. Blood samples were collected 1 hr. after the injections.

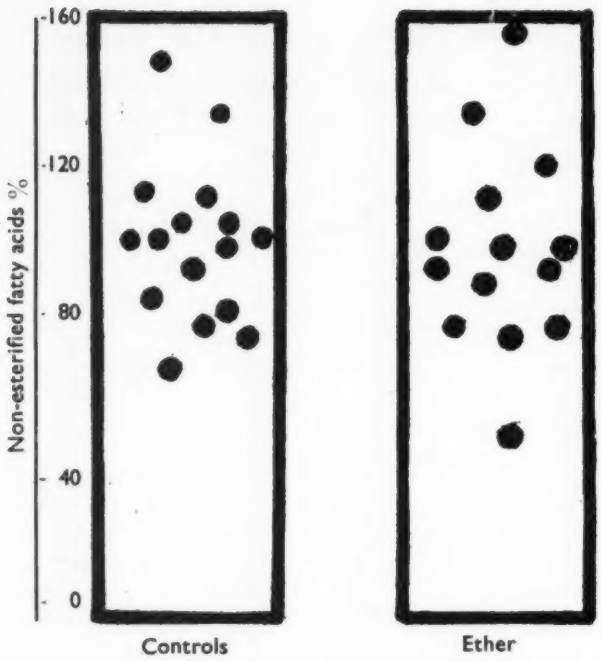

FIG. 2.—The effect of ether on the non-esterified fatty acid concentration in rat serum. 14 rats were anaesthetized with ether and 16 rats were untreated. Blood samples were collected at the end of 1 hr.

remained practically unchanged throughout the experiment. The concentrations of fatty acid esters had a tendency to fall, but as the standard errors were high the change cannot be considered significant.

Series 2

As can be seen from Fig. 1 a similar action of thiopentone on the concentration of non-esterified fatty acids and sugar was found in rats. The effects on fatty acid concentrations were highly significant ($P<0.001$, $t=6.359$); those on sugar significant ($P<0.05$, $t=2.534$; $P<0.01$ by the approximate rank test H).

Series 3

Fig. 2 presents the combined results of two experiments, the concentrations of non-esterified fatty acids being expressed as percentages of the mean value for the control group. It was found that 60 min. ether narcosis was without effect.

DISCUSSION

Our results show that during thiopentone anaesthesia the blood concentrations of non-esterified fatty acid in dogs and rats decline, whereas the blood sugar rises. Non-esterified fatty acids are important as intermediates in the utilization of fat, and as a mobile fraction necessary for fat resynthesis and deposition. In view of their significance study of their concentrations under different conditions has attracted much interest in recent years. When such experiments are carried out under thiopentone anaesthesia a primary response may be combined with side-effects of the thiopentone anaesthesia.

Results so far do not allow an unequivocal explanation of the mechanism leading to the drop of non-esterified fatty acids during thiopentone anaesthesia. One possible explanation might be a direct action of thiopentone on lipolysis in adipose tissue. On the other hand it is possible that during thiopentone anaesthesia the catabolism of fat is enhanced, and that the non-esterified fatty acids are in consequence removed more rapidly from the blood stream. However, a similar effect could also be accounted for by increased lipogenesis.

Blood sugar changes due to thiopentone anaesthesia have already been reported many times. Most authors report a slight increase of blood sugar (Blackberg and Hrubetz, 1937; Rašková and Vlček, 1947) while a decrease has been reported only after the cessation of anaesthesia (Benešová, Koutenská and Wenke, 1959).

An unequivocal interpretation of our results is not possible since among other factors our control animals, which were not under anaesthesia, were allowed to move, in contrast to the animals under anaesthesia. The question as to what extent the fall of non-esterified fatty acids is caused specifically by thiopentone, or whether it is a change primarily due to anaesthesia, is therefore left open at present. Our results with ether suggest that the former alternative is more reasonable.

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APOMORPHINE-INDUCED PECKING IN PIGEONS

BY

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(RECEIVED JANUARY 12, 1960)

Apomorphine produced persistent pecking in pigeons, the latent period, intensity and duration of which were related to the dose. The ED50 was estimated as 78.1 ±11.1 μg./kg. On chronic administration of apomorphine there was a significant decrease in latent period and weight which quickly returned to normal on stopping the drug. No conditioning and no tolerance were observed. The uncertain emetic effect of apomorphine in pigeons has been confirmed. Ten other centrally acting agents tested (caffeine, cocaine, 5-hydroxytryptamine, lysergic acid diethylamide, methamphetamine, morphine, nalorphine, pentylenetetrazol, strychnine, and yohimbine) failed to produce similar effects in pigeons.

While engaged in a comparative evaluation of anti-emetic drugs in pigeons (Gujral, Saxena and Dhawan, 1956), an attempt was made to produce emesis by apomorphine. It was seen that instead of emesis the drug constantly produced persistent pecking. The birds pecked rapidly at the tin floor and wires in the side and ceiling of the cage as if pulling off particles from them and swallowing them. They ignored any grain that was put in the cage. The effect came on very quickly, persisted for 0.5 to 1 hr. and was so compulsive that in many cases the beak got injured and started bleeding. The pigeons were depressed for some time after the pecking had stopped. This type of fruitless pecking was not seen in normal pigeons. No reference could be found in the literature about this effect of apomorphine in pigeons. Our interest in the problem was revived by a report of similar effects of apomorphine in pigeons by Koster (1957). It was decided to undertake a detailed study of apomorphine-induced pecking in pigeons and to see if a similar behaviour could be induced by some other drugs as well.

A preliminary report of this work was presented before the 1959 session of the Association of Physiologists and Pharmacologists of India at Poona (Dhawan and Saxena, 1960).

METHODS

Three hundred and fifty pigeons of either sex weighing between 200 and 400 g. were used in this study. The birds were housed in groups of 8 to 10 (regardless of sex) in large cages, and had free access to water and mixed grains. They were not used more than twice a week except when studying the effects of chronic administration. At the time of use they were placed separately in small wire mesh cages, and food and water were withdrawn. Experiments were carried out in a quiet room diffusely illuminated with daylight.

The drugs were dissolved in distilled water or suspended in a 4% homogenized gum acacia solution. Whenever possible solutions or suspensions were prepared immediately before use. Each dose was tested in at least one batch of 10 birds. Intramuscular injections were made in the pectoral muscles, intravenous injections through alar veins, and oral administration by letting the suspension drop in the mouth from a tuberculin syringe.

The birds were observed for at least 1 hr. after administration of drugs, and pecking was considered positive only if the bird pecked more than 10 times during this period. Active expulsion of crop contents was employed as the criterion for vomiting. The latent period was the period elapsing between the drug administration and the first act of pecking or vomiting respectively. The duration of pecking was the period during which at least 50% pigeons were pecking. Only an approximate estimate of the duration could, therefore, be made. The pecking was so rapid that it was difficult to count the exact number in each bird and an accurate gradation of the intensity could not be done.

RESULTS

Effects of Graded Doses of Apomorphine.— In a few preliminary experiments it was observed that there was little difference in the onset, duration or intensity of pecking following intravenous or intramuscular administration of apomorphine. Hence it was decided to give the drug intramuscularly. Graded doses of apo-

morphine hydrochloride (British Drug Houses) from 12.5 to 12,000 μg./kg. were administered and the effect was assessed in terms of (a) the percentage of birds affected, (b) the latent period, (c) the duration of pecking, (d) the intensity of pecking and (e) the presence or absence of vomiting.

The final regression line obtained by the method of Bliss (1935a and b) is shown in Fig. 1. It was tested for goodness of fit by the χ^2 test (Finney, 1952) and found to be homogeneous. The ED50 was found to be 78.1 ± 11.1 μg./kg. When higher dose levels of apomorphine were tested, an occasional bird was met with which did not respond at certain dose levels (Table I, 600, 800 and 2,400 μg./kg. respectively). With still larger doses, however, all the birds responded. The latent period progressively decreased as the dose of apomorphine was increased. The duration of pecking also was more in batches receiving higher doses of apomorphine. It was not possible quantitatively to evaluate the severity of pecking in various groups, but it was observed that pecking was more vigorous with increasing doses. None of the pigeons vomited in response to doses of apomorphine below 2,400 μg./kg. Even in higher doses the response was erratic (see effects of 4,800 μg./kg., Table I).

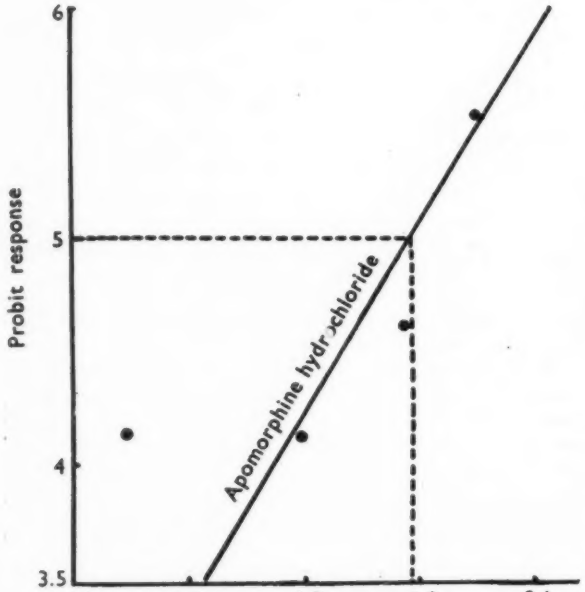

FIG. 1.—Showing the final regression line for apomorphine-induced pecking in pigeons. The ordinate shows the transformed probit values for percentage of birds responding. The ED50 value has been marked. The points refer to the experimental data obtained.

TABLE I

THE EFFECTS OF APOMORPHINE ON PIGEONS

Groups of pigeons were treated with apomorphine in doses ranging from 12·5 μg. to 12 mg./kg. The incidence of pecking and vomiting was recorded. A pecking frequency greater than 10/min. was considered a positive response. The duration was the period during which 50% or more of the birds were pecking. The latent period was the time between drug administration and the onset of pecking or vomiting respectively. The mean latent period for pecking was calculated for each group and the standard error is given in parentheses.

Apomorphine μg./kg.	No. of Pigeons Tested	% Pecking	Duration (min.)	Mean Latent Period in min.	Vomiting	
					%	Latent Period (min.)
12·5	10	0				
25	10	20	7	6·0 (1·0)		
50	10	20	7	6·0 (1·0)		
75	20	35	8	8·0 (1·1)		
100	10	70	25	6·0 (0·8)		
150	10	100	35	6·1 (0·9)		
200	10	100	40	3·3 (0·4)		
250	20	100	40	4·0 (0·4)		
400	10	100	40	5·5 (0·8)		
600	7	86	40	6·5 (0·9)		
800	8	88	60	4·0 (0·6)		
2,400	10	90	105	4·3 (1·2)	10	4
3,600	10	100	120	2·4 (0·3)		
4,800	10	100	120	2·0 (0·5)	70	18
4,800	10	100	120	2·1 (0·8)		
6,000	10	100	120	2·0 (0·3)	40	18
12,000	10	100	120	1·4 (0·4)	20	10

Effects of Chronic Administration.—In one batch of 10 pigeons effects of daily administration of 250 μg./kg. (approximately $2 \times$ ED99) apomorphine for 15 days were studied. The results obtained are shown in Fig. 2. The percentage of birds affected and the duration of pecking were unaltered by daily administration. There was, however, a significant decrease ($P = 0.05$) in the latent period from the third day of the start of the experiment (Fig. 2). After stopping apomorphine the latent period returned towards the control level and a week later no significant difference from the initial value could be seen (Fig. 2). The weights of the birds also started falling, and by the tenth day a significant ($P = 0.05$) loss in weight was observed. After stopping the apomorphine,

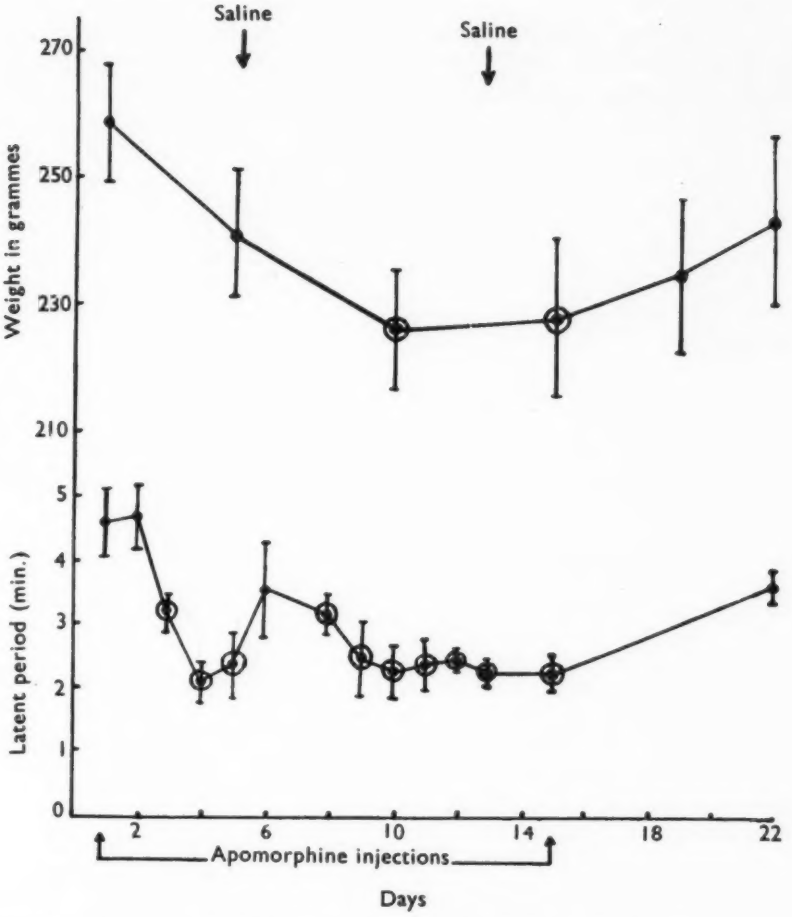

FIG. 2.—Showing the effect of chronic intramuscular injection of apomorphine (250 μg./kg. daily) on the mean weight and latent period for pecking of a group of 10 pigeons. The points surrounded by a circle (O) differ significantly (P=0.05) from the starting values. The vertical lines represent standard errors of the mean. At Saline a sham procedure was performed with saline injection. Note the quick recovery on stopping apomorphine.

the weight returned towards the initial level. The difference in weight on the 19th and 22nd day is not significant statistically from control values. A sham procedure with normal saline injections was performed on 2 occasions (fifth and thirteenth day) during the study (Fig. 2). There was, however, no conditioned pecking after saline injection.

Effects of Various Factors on Pecking.—Hypoglycaemia produced by fasting, administration of insulin (0.2 unit/kg. injected subcutaneously) or tolbutamide (100 mg./kg. orally) failed to alter the incidence, duration or latency of subsequent pecking induced by 250 μg./kg. of apomorphine. The response to apomorphine was also unaffected by previous administration of a carbonic anhydrase inhibitor, acetazoleamide (150 mg./kg. orally). The results have been summarized in Table II.

Intensity of pecking was considerably reduced if the room was made dark and in some cases the pecking stopped altogether in darkness. The pecking could be interrupted by vigorous thumping on the table, loud noise, etc., but was always resumed with the same intensity after a few minutes. Occurrence of vomiting also subdued

TABLE II

THE EFFECTS OF VARIOUS PROCEDURES ON APOMORPHINE (250 μG./KG.) INDUCED PECKING IN PIGEONS. THE CONTROL GROUP RECEIVED APOMORPHINE ALONE

P values refer to difference between treated and control groups. L.P.=latent period.

Procedure or Drug	Number of Birds	Apomorphine Challenge After	% Positive Responses to Apomorphine	Mean L.P. in min. ±S.E.	P	Approximate Duration in min.
Control	10		100	4·9±0·5		40
Fasting	10	24 hr.	100	4·6±0·4	0·5	40
Insulin	10	0·5 ,,	100	5·0±1·0	0·1	40
Tolbutamide	10	3 ,,	100	4·9±1·0	0·9	40
Acetazoleamide	10	3 ,,	90	3·5±0·44	0·5	40

pecking for some time. Pecking could be induced even in pigeons where both eyes had been destroyed.

Testing of Other Drugs for Pecking Effect.— Ten centrally acting agents, caffeine citrate (100 mg./kg.), cocaine hydrochloride (24 mg./kg.), 5-hydroxytryptamine creatinine phosphate (250 and 1,000 μg./kg.), lysergic acid diethylamide (25 and 100 μg./kg.), methamphetamine hydrochloride (5 mg./kg.), morphine tartrate (1 and 10 mg./kg.), nalorphine hydrochloride (1 and 20 mg./kg.), pentylenetetrazol (25 mg./kg.), strychnine hydrochloride (500 μg./kg.) and yohimbine hydrochloride (75, 150 and 600 μg./kg.), were tested for their ability to produce pecking in pigeons. All drugs were given intramuscularly. Occasional pecking (frequency never more than 10/hr.) was observed in some of the birds receiving yohimbine (150 μg./kg.) and cocaine. Lysergic-acid-diethylamide-treated birds appeared unusually sedated as contrasted with its excitatory effect in other species. Many of these agents produced emesis in some of the pigeons.

DISCUSSION

The results obtained clearly demonstrate that pecking is a characteristic and well defined behavioural disturbance induced by apomorphine in pigeons. The phenomenon can be produced by such minute amounts that the procedure might be useful for detecting small amounts of apomorphine. Results of chronic administration (Fig. 2) suggest that tolerance does not develop to this effect of apomorphine. There is a definite facilitation after a few days which may be due to accumulation of the drug in the body or due to a sensitization of the receptors concerned. That facilitation is not due to conditioning is shown by the fact that sham procedures with saline injections are ineffective in eliciting a pecking response (Fig. 2). The loss in weight seen during the chronic administration of apomorphine could be due either to the excessive muscular activity involved in the pecking or to some toxic effect of the drug itself.

It is difficult to explain the mechanism of this effect of apomorphine on the basis of the existing data. The reaction does not seem to be identical with pre-emetic nausea of other emetic agents. Our data confirm the findings of several other workers that apomorphine is an uncertain emetic in pigeons (Madjarek and Stern, 1956; Zeehuisen, 1895). This inability of apomorphine to produce consistent emesis in pigeons strongly suggests that the chemoreceptor trigger zone for emesis (Wang and Borison, 1950) is either rudimentary in pigeons or is qualitatively different from that in other species. The results of Hanzlik and Wood (1929) with digitalis emesis in pigeons also support this contention. Again tolerance has been reported to emetic action of apomorphine in other species (Co Tui, 1931), while no tolerance is observed to its pecking effect. Finally, large doses of apomorphine are capable of preventing apomorphine-induced vomiting in dogs (Dordoni, 1951; Hatcher, 1924; Koppanyi, 1930; Leake, 1922). The pecking effect is, however, more intensive with increasing doses of apomorphine.

Koster (1957) has suggested that the disturbance might be a feeding hallucination. However, some known hallucinogenic agents (lysergic acid diethylamide, cocaine, yohimbine, nalorphine) tested fail to produce pecking. The response is unaffected by hypoglycaemia induced by fasting, insulin or tolbutamide. Hypoglycaemia is known to increase excitability of nervous structures (Kety, Polis, Nadler, and Schmidt, 1948).

Similarly decreased brain excitability produced by acetazoleamide (Millichap, Woodbury, and Goodman, 1955) has not altered the pecking response. The response is also unaffected by prior administration of azacyclonol, an antihallucinogenic agent (unpublished observations).

It is possible that the phenomenon may be identical with chewing movements produced by apomorphine in rat (Du Toit and Christensen, 1948) and rabbit (Brucke, Petsche, Sailer, and Stumpf, 1957). Brucke *et al.* (1957) have shown that there is a pronounced and long-lasting desynchronizing effect on precentral cortical electroencephalograph accompanying it in rabbits. A similar electroencephalographic pattern is produced by methamphetamine. Methamphetamine is, however, unable to produce a pecking response in pigeons. Some valuable data about the mechanism of apomorphine pecking may be obtained by studying the effect of various groups of pharmacological agents on this response. Work on these lines is already in progress and will be published in a separate communication.

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THE BRONCHOCONSTRICTOR ACTION OF BRADYKININ IN THE GUINEA-PIG

BY

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Bradykinin was found to be a potent bronchoconstrictor agent in the guinea-pig anaesthetized with urethane. This action was not affected by vagotomy, or by treatment of the animal with mepyramine, atropine, lysergic acid diethylamide, or cortisone. Adrenaline and isoprenaline suppressed the bronchoconstrictor responses to bradykinin and histamine. Small doses of acetylsalicylic acid, however, suppressed only that to bradykinin. Bradykinin also produced bronchoconstriction in the isolated perfused lungs of the guinea-pig. The closely related peptide, wasp kinin, was also a potent bronchoconstrictor.

Bradykinin is known to affect smooth muscles of intestine, uterus and blood vessels. When the action of bradykinin on bronchiolar muscle of the guinea-pig was investigated, it was found to be a potent bronchoconstrictor agent. A preliminary report of this observation has already been made (Collier, Holgate, Schachter and Shorley, 1959), and the present account describes the findings in detail.

METHODS

Materials.—Bradykinin was prepared by the action of crystalline trypsin on heated bovine serum globulin and was purified chromatographically (Lockhart and Jones, personal communication). The potencies of the various samples used, assayed on guinea-pig ileum against a standard preparation (Rocha e Silva, Beraldo and Rosenfeld, 1949), ranged from 7 to 900 units/mg. After the work described below was completed, Elliott, Lewis and Horton (1960) kindly provided us with a sample of pure bradykinin; and comparison with our reference preparation indicated that about 12,000 to 12,500 units were equivalent to 1 mg. pure bradykinin, or that 1 unit was equivalent to about 80 ng. Wasp kinin was prepared by washing dried venom glands with alcohol, extracting the kinin from the residue with glacial acetic acid and precipitating it with ether (Holdstock, Mathias and Schachter, 1957). Chymotrypsin and trypsin (Armour) were crystalline preparations. The following salts were used: acetylcholine chloride, adrenaline hydrochloride, atropine sulphate, DL-cysteine hydrochloride, 5-hydroxytryptamine creatinine sulphate, isoprenaline hydrochloride, lysergic acid diethylamide tartrate, mepyramine maleate, sodium phenylbutazone. All weights are expressed in terms of the active acid or base. Acetylsalicylic acid was suspended in 5% gum acacia for intraperitoneal administration. For intravenous injection, 10 parts by weight of acetylsalicylic acid were mixed with 3 parts of calcium carbonate and 1 part of citric acid, and the mixture dissolved in water immediately before use. Cortisone, as the alcohol, was suspended in saline for intramuscular or subcutaneous injection. Amidopyrine was administered as the base.

Whole Animal Preparations.—The method was based on that of Konzett and Rössler (1940). Guinea-pigs (300 to 700 g.) were anaesthetized with urethane (1.0 to 1.25 g./kg. i.p.). Further urethane was given intraperitoneally when necessary to maintain suppression of spontaneous respiratory movements. The trachea was cannulated and the lungs inflated with air by a miniature Starling pump operating on a partially-closed circuit (5 to 10 ml. stroke volume, generally at 72 strokes/min.). A sidearm from the cannula permitted some air to escape through a water valve offering a resistance of 10 cm. water, and the escaping air operated a piston recorder. When resistance of the lungs to inflation increased, more air passed through the sidearm, causing a greater excursion of the recording lever. Solutions of drugs in saline were injected at 5 to 10 min. intervals through a cannula in the external jugular vein and 0.05 to 0.4 ml. washed in with 0.4 ml. heparinized saline (10 units/ml.). After a marked response of the lungs, increased resistance to inflation might persist for some time. This was overcome by momentarily

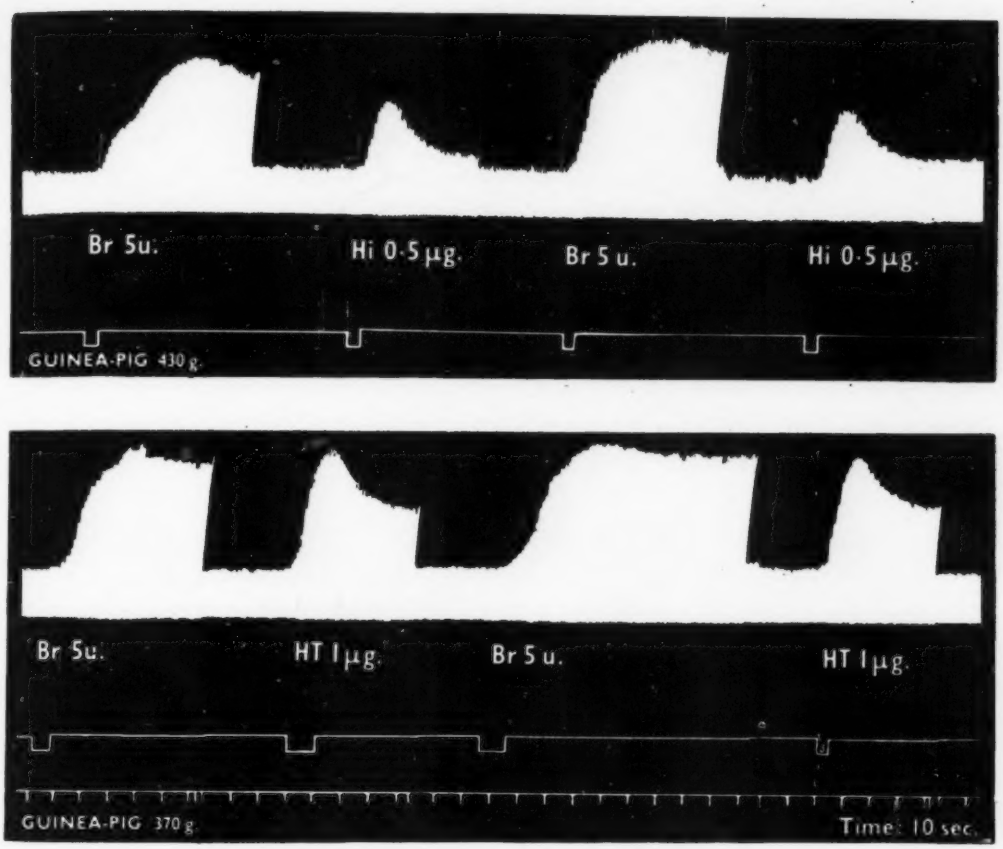

FIG. 1.—Resistance to inflation of guinea-pig lungs *in vivo*. Increased resistance caused by bradykinin (Br), histamine (Hi), and 5-hydroxytryptamine (HT). Doses were administered intravenously at 10 min. intervals.

closing the sidearm of the cannula, thus more forcibly inflating the lung.

Isolated Perfused Lungs.—Isolated lungs were perfused with Tyrode solution through a cannula in the pulmonary artery. Drugs were injected into the perfusion fluid, and inflation and recording were as in whole animal preparations.

RESULTS

Action of Bradykinin

In Whole Animals.—In the course of experiments on 87 guinea-pigs, prepared for recording resistance of the lungs to inflation, bradykinin was administered intravenously in doses ranging from 1.25 to 80 units. In all animals, bradykinin increased resistance to inflation, doses of 2.5 to 10 units normally being effective (Fig. 1). This figure shows that the response to bradykinin differs slightly from those to histamine and 5-hydroxytryptamine in its slower onset and longer persistence at or near peak level. In four experiments, cutting the vagi in the neck produced no significant alteration of the pulmonary response to bradykinin. In a number of guinea-pigs, the chest wall was opened to permit visual inspection of the lungs. This procedure also failed to influence significantly the recorded response to bradykinin. We observed that effective doses of bradykinin or histamine invariably reduced lung excursion. After larger doses of either drug, there appeared in the lungs cyanotic patches, some of which persisted. These patches could be removed by closing the sidearm of the tracheal cannula and so forcing air into the lungs.

When bradykinin was given repeatedly, refractoriness usually developed. This was more noticeable than it was with histamine, to which refractoriness developed less readily or not at all.

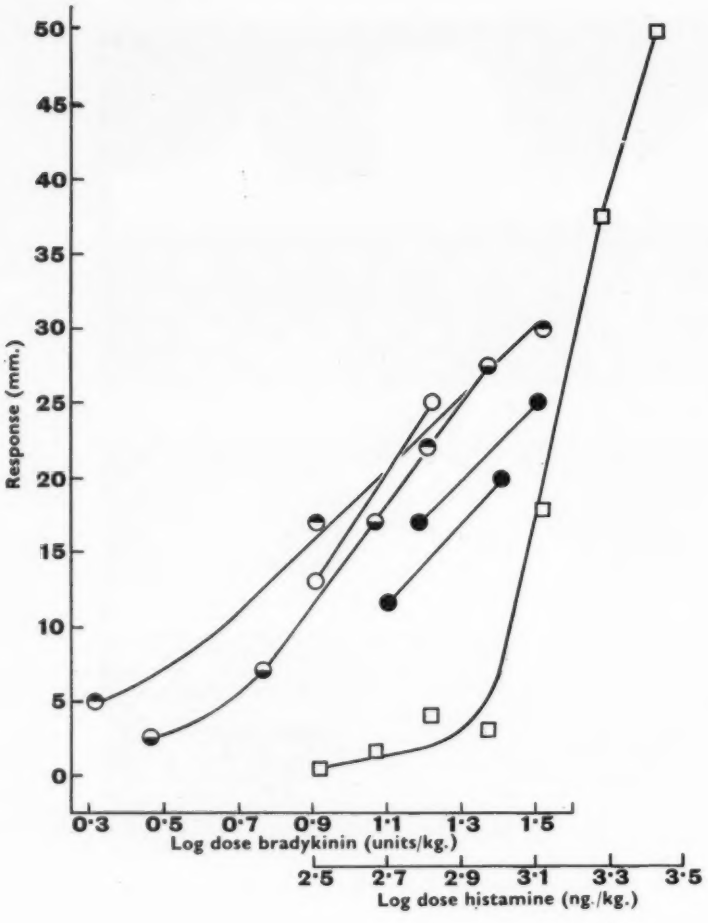

FIG. 2.—Resistance to inflation of guinea-pig lungs *in vivo*. Dose-response curves of histamine (□—□) and four samples of bradykinin (●—●, 7 u./mg.; ◓—◓ and ◒—◒, 200 u./mg.; ○—○, 900 u./mg.).

Before refractoriness became serious, however, increasing the dose of bradykinin produced increased resistance to inflation, enabling dose-response curves to be obtained. In Fig. 2 are illustrated a number of such curves, each from a different animal, obtained with samples of bradykinin assayed at 7, 200 and 900 units/mg. on the guinea-pig ileum. All curves were less steep than that of histamine in a similar experiment. It is evident from Fig. 2 that the samples of bradykinin containing 200 and 900 units/mg. showed the same relative potencies on lung as on ileum. In order to ascertain whether the potencies of the 7 and 200 unit samples also ran parallel on lung and ileum, we administered doses of 5 and 10 units of each to alternate guinea-pigs in a series of 12 animals. Statistical analysis of the results showed that the relative potency of these preparations on lung did not differ significantly from their activity ratio on the guinea-pig ileum.

The activity of bradykinin on the lungs was abolished by incubating it with chymotrypsin (25 μg. in 0.1 ml. saline + 10 units bradykinin in 0.1 ml. saline) for 10 min. at room temperature. Incubation of bradykinin with trypsin in the same way, however, did not abolish its activity. Incubation with guinea-pig serum (0.05 ml. + 5 units bradykinin in 0.05 ml. saline) for 30 min at 35° also inactivated bradykinin. This effect was reduced if 1 mg. cysteine was added to the mixture incubated (5 units bradykinin in 0.05 ml. saline + 0.1 ml. serum). Records of these experiments are shown in Fig. 3.

In Isolated Perfused Lungs.—Bradykinin and histamine increased the resistance to inflation of isolated perfused lungs, as in whole animals (Fig. 4). To evoke responses from isolated lungs, larger amounts of both agents were required, but their relative potencies were unchanged.

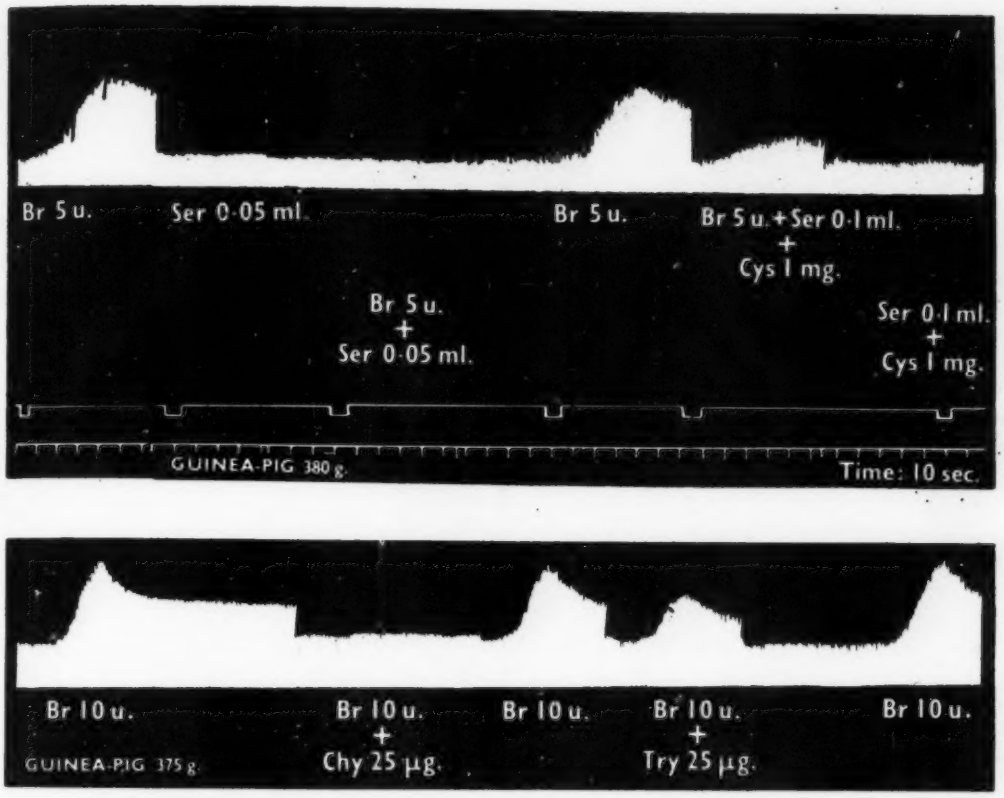

FIG. 3.—Resistance to inflation of guinea-pig lungs *in vivo*. Inactivation of bradykinin on incubation with guinea-pig serum (Ser) or with chymotrypsin (Chy), but not on incubation with trypsin (Try). Protection of bradykinin by cysteine (Cys).

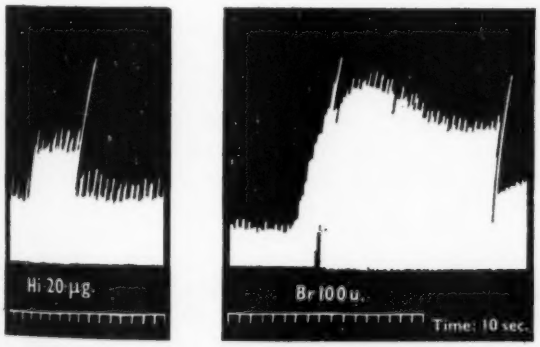

FIG. 4.—Resistance to inflation of guinea-pig lungs *in vitro*. Responses of isolated perfused lungs to histamine and bradykinin. In the histamine experiment, lungs were inflated at 60 strokes/min.; in the bradykinin experiment, in another preparation, at 72 strokes/min.

Action of Wasp Kinin

Intravenous injection of wasp kinin in 10 guinea-pigs increased resistance to inflation. In the experiment illustrated in Fig. 5, the partially purified preparation of wasp kinin exhibited about one-third of the potency of histamine on a weight basis. Like the response to bradykinin, that to wasp kinin was slower in onset and more sustained than the histamine response. As with bradykinin, opening the chest wall did not significantly alter the effectiveness of wasp kinin, which also produced in the lungs cyanotic patches that could be removed by more forcible inflation.

Effects of Drugs on the Response to Bradykinin

The action of bradykinin was unaffected by intravenous doses of mepyramine or lysergic acid diethylamide that abolished the responses to equivalent doses of histamine or 5-hydroxytrypt-

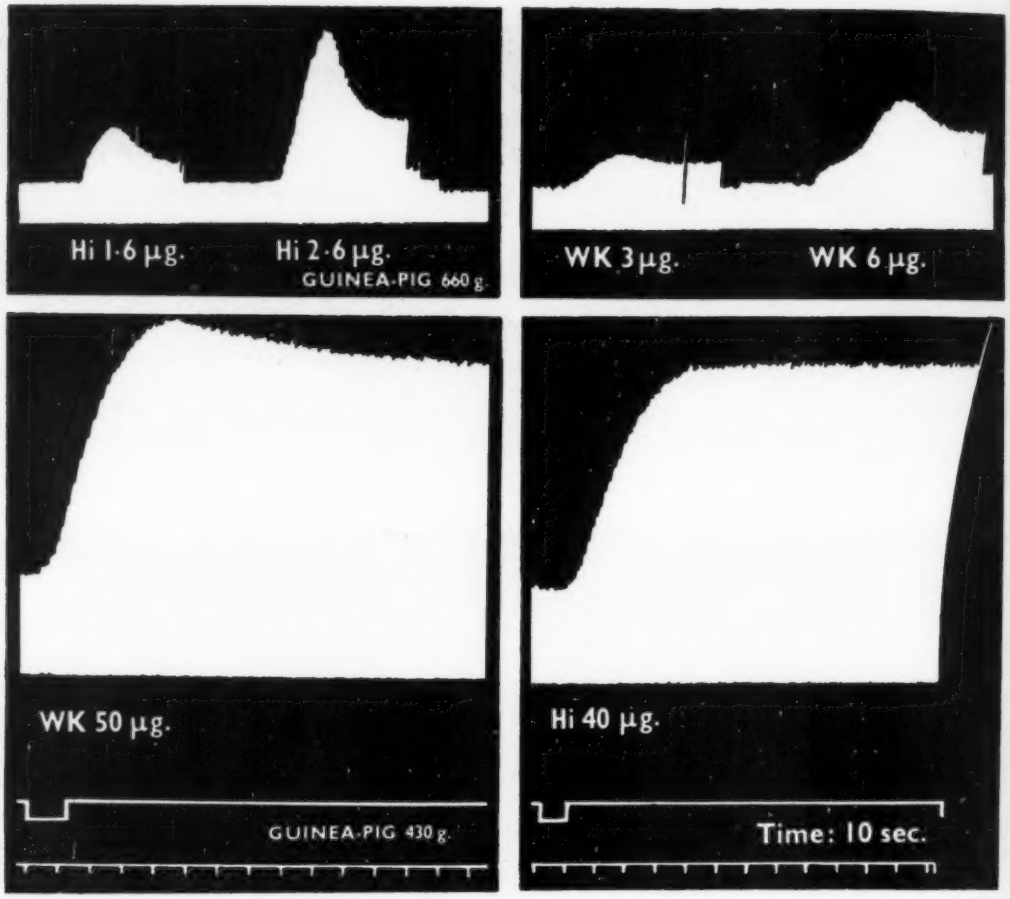

FIG. 5.—Resistance to inflation of guinea-pig lungs *in vivo*. Increased resistance caused by wasp kinin (WK) and histamine.

amine. In the experiment illustrated in Fig. 6, small doses of antagonists were used ; but in other experiments as much as 0.5 mg./kg. mepyramine failed to affect the response to bradykinin. Doses of atropine that suppressed the response to acetylcholine likewise did not affect that to bradykinin.

Intravenous injection of adrenaline (2 μg./kg.) or isoprenaline (0.6 μg./kg.) given together with histamine or bradykinin completely suppressed their actions on the lungs. The response to adrenaline was of brief duration, and, a few minutes after its injection, responses to histamine and bradykinin could again be elicited.

Acetylsalicylic acid, injected intraperitoneally or intravenously, completely suppressed the response to bradykinin without materially affecting those to histamine or 5-hydroxytryptamine (Fig. 7). This effect could be obtained with doses as low as 2 mg./kg. when given intravenously. Amidopyrine and sodium phenylbutazone, given intravenously, had comparable effects, but possessed only about one-half to one-eighth the potency of acetylsalicylic acid. Acetylsalicylic acid also suppressed the response to wasp kinin.

Cortisone was administered subcutaneously to a guinea-pig in three successive daily doses of 2 mg./kg. After the last dose, the animal was prepared for recording pulmonary resistance to inflation, and injected with bradykinin and histamine. The responses to these two agents were unaffected by pre-treatment with cortisone. In another experiment pre-treatment of a guinea-pig, with successive intramuscular doses of 2, 10 and 25 mg./kg. cortisone, administered within 2.5 hr., failed to influence the bronchoconstrictor action of bradykinin or histamine.

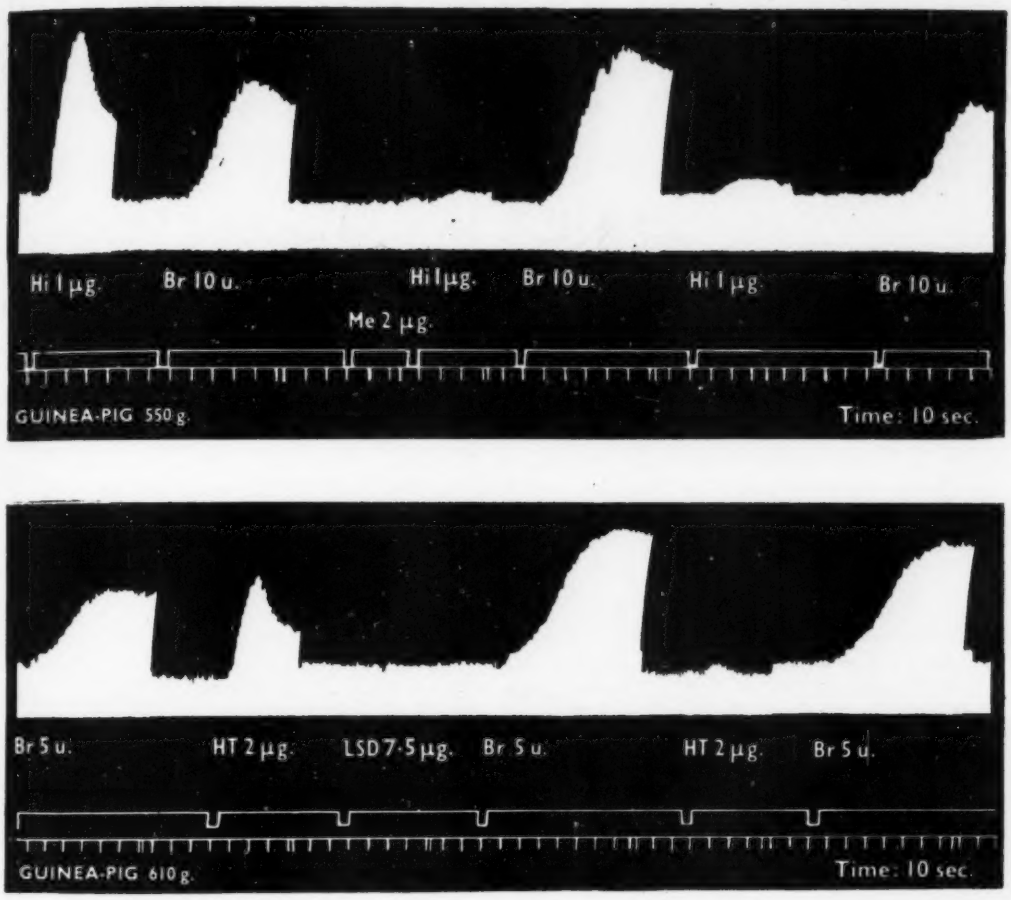

FIG. 6.—Resistance to inflation of guinea-pig lungs *in vivo*. Failure of mepyramine maleate (Me) and lysergic acid diethylamide (LSD) to reduce response to bradykinin.

DISCUSSION

Increased resistance of the lungs to inflation after bradykinin might be due to (1) increased resistance of the chest wall; (2) an increase of fluid in the lungs; or (3) bronchoconstriction. The first possibility can be eliminated because the response occurs after opening the chest wall and in the isolated lungs. The second possibility is very unlikely because (*a*) the response was obtained in the isolated lung without obvious change in the rate of flow of perfusion fluid, and (*b*) at the height of response the lung could sometimes be seen to be deflated, with no sign of fluid engorgement. We are left with the conclusion that the effect of bradykinin is due to bronchoconstriction. This is supported by the close resemblance of the response, except in small details of time-course, to that to histamine, which is an established bronchoconstrictor. The view that the effect of bradykinin is due to bronchoconstriction is also supported by its suppression by adrenaline and isoprenaline, which are well known to dilate bronchioles. The same evidence was obtained for a bronchoconstrictor action of wasp kinin, except that it was not tested on isolated lungs.

Since 1 unit is equivalent to about 0.08 µg. pure bradykinin, it is evident from Fig. 1 that, weight for weight, bradykinin is at least as potent as histamine. Wasp kinin, of which an impure preparation was used, must also be of this order of activity. In terms of molecules, therefore, the two peptides must rank among the most potent known stimulants of bronchiolar smooth muscle. It may well be that other natural peptides also possess bronchoconstrictor properties.

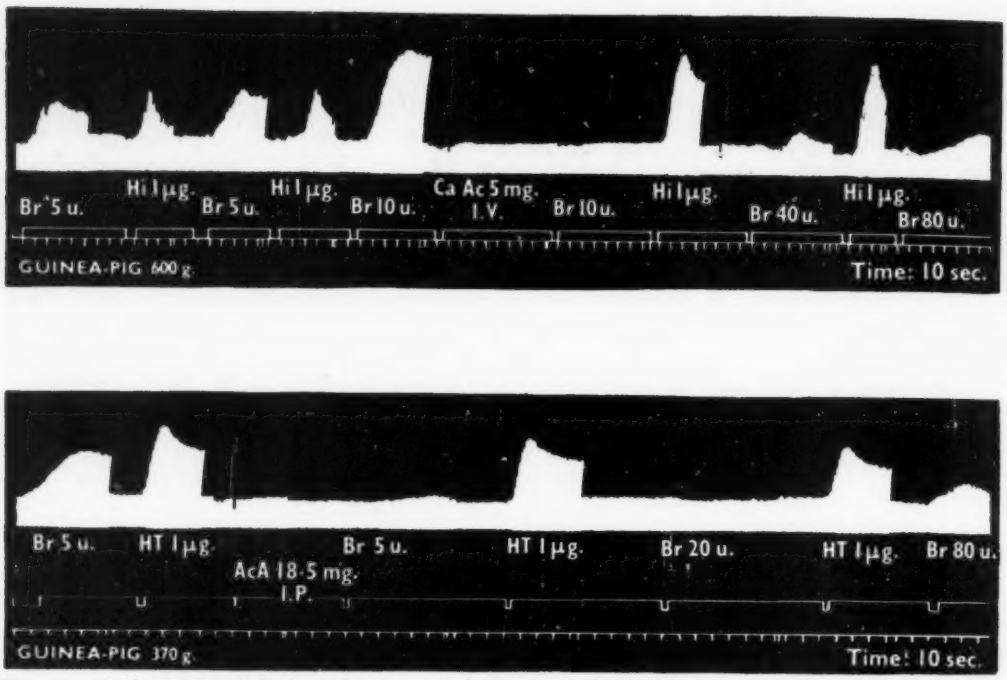

FIG. 7.—Resistance to inflation of guinea-pig lungs *in vivo*. Suppression of response to bradykinin by aspirin. Ca Ac=calcium acetylsalicylate, dose as acid. AcA=acetylsalicylic acid. Dose of AcA given 4 min. before bradykinin.

The relatively slow onset of the response to bradykinin allows the possibility that it acts through release of histamine or other substance. This possible explanation is made unlikely by the fact that mepyramine, atropine and lysergic acid diethylamide, in doses which antagonized known endogenous bronchoconstrictor substances, did not suppress the response to bradykinin.

Until a pure preparation is available, it will be impossible to state with certainty that the principle which constricts bronchiolar muscle is the same as that which contracts guinea-pig ileum. None the less, two facts strongly suggest that the two principles are identical. First, throughout purification, the potencies of bradykinin preparations on guinea-pig ileum and on lung ran parallel. Secondly, like the ileum-stimulating principle (Werle, 1955; Schachter, 1960), the bronchoconstrictor principle is inactivated by guinea-pig serum and by chymotrypsin, though not by trypsin, and is stabilized by cysteine.*

The experiments reported also raise the question whether bradykinin or a related peptide participates in human asthma. We have no evidence concerning this at present; but the amounts of kinin that might be present in plasma in certain circumstances are sufficient to allow the question to remain open.

The action of acetylsalicylic acid in suppressing the response to bradykinin is interesting for several reasons. First, this is a potent *in vivo* effect of acetylsalicylic acid which occurs at concentrations in the blood that are much lower than those reached in man during salicylate therapy. Also, amidopyrine and phenylbutazone, two other antirheumatic substances which were tested, suppressed the bradykinin response. This raises the question whether suppression of a response to bradykinin or to a related peptide contributes to the antirheumatic action of non-steroid agents.

We wish to thank Dr. I. M. Lockhart and Mr. W. A. Jones for supplies of bradykinin, Dr. B. T. Warner for statistical advice and Miss L. Deeming, Miss S. Horwood-Barrett and Miss M. Nicholas for technical assistance. We also wish to thank Dr. D. F. Elliott, Dr. G. P. Lewis, and Dr. E. W. Horton for a sample of pure bradykinin.

* *Note added in proof.* Subsequent experiments with pure bradykinin by Elliott, Lewis, and Horton, and by ourselves, confirm that the pure material exerts the expected bronchoconstrictior action and that this is specifically suppressed by aspirin.

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POTENTIATION OF SULPHADIMIDINE BY 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE AND OTHER 6,7-DISUBSTITUTED 2,4-DIAMINOPTERIDINES AGAINST *EIMERIA* INFECTIONS OF CHICKS

BY

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Six 2,4-diaminopteridines substituted in the 6 and 7 positions were tested as potentiators of sulphadimidine against *Eimeria tenella*. 2,4-Diamino-6,7-di-isopropylpteridine and 2,4-diamino-1′-propylindolo(2′,3′:6,7)pteridine were selected for further study. Both potentiated sulphadimidine against *E. tenella* and the di-isopropylpteridine potentiated it against *E. necatrix*. A 10% mixture of the di-isopropylpteridine with sulphadimidine (or other sulphonamides) appears to offer advantages in the treatment of *E. tenella* and still more of *E. necatrix*.

Coccidia are protozoal parasites of the alimentary tract of fowls. *Eimeria tenella* is the cause of caecal coccidiosis, a common and often lethal disease of young chickens. There are also several forms of intestinal coccidiosis which are becoming increasingly important in Britain as diseases of older fowls. The different forms of intestinal coccidiosis are caused by different species. A form of intestinal coccidiosis caused by *E. necatrix* is often responsible for a high death rate in flocks. Owing to their economic importance, the species *E. tenella* and *E. necatrix* were selected as the infective agents in the present work.

Certain sulphonamides exhibit pronounced anticoccidial properties and have been used in the control of field outbreaks of caecal coccidiosis for a number of years. Horton-Smith and Taylor (1945) showed that sulphadimidine could be used successfully to treat established infections of *E. tenella* in chickens. Later Horton-Smith and Boyland (1946) and Waletzky and Hughes (1946) found that its inhibitory effect could be reversed by *p*-aminobenzoic acid. These observations suggested that "more potent inhibitors of this parasite (*E. tenella*) might be found among other more active structures known to interfere with the *p*-aminobenzoic acid-folic acid sequence" (Lux, 1954). Lux obtained encouraging results when 2,4-diaminopyrimidines or 2,4-diamino-dihydro-*s*-triazines were mixed with sulphonamides, pyrimethamine being a particularly effective synergist. Joyner and Kendall (1955, 1956) confirmed this observation and showed that full therapeutic control of caecal coccidiosis was obtained with 0.005% pyrimethamine mixed with 0.05% sulphadimidine. Kendall and Joyner (1958) extended this work and showed that 2,4-diamino-6,7-di-isopropylpteridine and other folic acid antagonists potentiated sulphadimidine against *E. tenella*.

Collier, Campbell and Fitzgerald (1950) first described 2,4-diamino-6,7-di-isopropylpteridine as a vibrostatic agent, which potentiated sulphaguanidine in antibacterial activity, but not in toxicity to mice. Collier and Waterhouse (1950) described the antagonism between this pteridine and folic acid, and Collier and Phillips (1954) the antagonism between folinic acid and the pteridines used in the present investigation. Pteridines of this type were also shown to be active inhibitors of *Streptococcus faecalis* and *Staphylococcus aureus* (Collier and Waterhouse, 1952) and of *Plasmodium gallinaceum* (Bishop, 1954) and *P. berghei* (Thurston, 1954). The pteridines selected for the present trial were those shown most active in the above experiments.

We describe below extended observations showing potentiation of sulphadimidine by admixture with pteridines against *E. tenella*. We also describe the successful treatment with

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mixture of 2,4-diamino-6,7-di-isopropylpteridine and sulphadimidine of chicks infected with a strain of *E. necatrix* not particularly susceptible to sulphonamides alone (Horton-Smith and Long, 1959).

METHODS

Chemicals.—Sulphadimidine and the six 2,4-diamino-pteridines were administered as bases and *p*-amino-benzoic acid as the acid. All the pteridines were substituted in the 6 and 7 positions, and are listed

TABLE I

EFFECTS OF SIX DIFFERENT PTERIDINES ON THE SULPHADIMIDINE TREATMENT OF CHICKS INFECTED WITH *E. TENELLA*

Groups 1 to 7 were given diets containing 0·05% sulphadimidine, and groups 1 to 6 diets containing 0·005% of the appropriate pteridine.

Group	Treatment	Experiment 1	
		No. of Birds	No. of Deaths
1	2,4-Diamino-6,7-di-isopropylpteridine+sulphadimidine	12	4
2	2,4-Diamino-6,7-di(cyclohexylmethyl)pteridine+sulphadimidine ..	12	8
3	2,4-Diamino-1′-methylindolo(2′,3′: 6,7)pteridine+sulphadimidine ..	12	8
4	2,4-Diamino-1′-propylindolo(2′,3′: 6,7)pteridine+sulphadimidine ..	12	2
5	2,4-Diamino-6,7-dibutylpteridine+sulphadimidine	12	9
6	2,4-Diamino-6,7-dipentylpteridine+sulphadimidine	12	9
7	Sulphadimidine alone	12	7
8	No treatment	12	12

TABLE II

EFFECTS OF 2,4-DIAMINO-6,7-DI-ISOPROPYL- AND 2,4-DIAMINO-1′-PROPYLINDOLO(2′,3′: 6,7)-PTERIDINE ON THE SULPHADIMIDINE TREATMENT OF CHICKS INFECTED WITH *E. TENELLA*

Pteridine	Pteridine % in Diet	Sulpha-dimidine % in Diet	Experiment 2		Experiment 3		Total		
			No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
Di-isopropyl	0·00625	0·00625	9	7			9	7	78
	0·00125	0·0125	9	6			9	6	67
	0·0025	0·025	9	7	9	2	18	9	50
	0·005	0·05	9	1	9	2	18	3	17
	0·01	0·1			9	0	9	0	0
	0·005	—	9	7			9	7	78
	0·01	—			9	7	9	7	78
Propylindolo	0·000625	0·00625	8	6			8	6	75
	0·00125	0·0125	8	5			8	5	63
	0·0025	0·025	8	3	9	1	17	4	24
	0·005	0·05	8	1	9	1	17	2	12
	0·01	0·1			9	0	9	0	0
	0·005	—	9	7			9	7	78
	0·01	—			9	8	9	8	89
—	—	0·05	9	6	9	5	18	11	61
	—	0·1			9	2	9	2	22
	—	—	9	6	9	7	18	13	72

in Table I. The methods of synthesis of these pteridines were described by Campbell, Dunsmuir, and Fitzgerald (1950).

Tests in Chicks.—Rhode Island Red × White Leghorn chicks aged 8 to 16 days at time of infection were used in the experiments. Groups of chicks were maintained in electrically heated metal brooders with wire floors and were kept for 13 to 18 days after infection with oocysts. *E. tenella* and *E. necatrix* cultures were derived from single oocyst infections. Cultures of oocysts used for infecting chicks in these experiments were freshly collected where possible and never stored for longer than 45 days. The infective dose was 50,000–100,000 oocysts of either species. Medication commenced 48 hr. after infection with oocysts and continued for 3 days, that is, until the 5th day of the infection.

Full details of methods used in chemotherapy experiments and the composition of the diet have already been described (Horton-Smith and Long, 1956, 1959). In toxicity tests drugs were administered in the diet to uninfected birds of the same age and type, food intake being measured.

Results

Therapeutic Tests against E. tenella

Comparison of Pteridines as Potentiators of Sulphadimidine.—In a preliminary experiment involving 96 chickens, the six pteridines listed in Table I were administered at a concentration of 0.005% in the diet, together with sulphadimidine at 0.05%. Only those groups of birds receiving the di-isopropyl- and propylindolo-pteridine in addition to sulphadimidine had lower mortalities than the control group receiving sulphadimidine alone (Table I).

The effects of these two active pteridines mixed with sulphadimidine were compared in two experiments involving 203 chicks (Table II). In these experiments the pteridines alone were ineffective, but mixtures of either pteridine and sulphadimidine were effective if given in sufficient concentration in the diet. The propylindolopteridine appeared slightly more active than the di-isopropylpteridine.

TABLE III

SUMMARY OF FOUR EXPERIMENTS ON 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE AND SULPHADIMIDINE AND THEIR MIXTURES IN THE TREATMENT OF CHICKS INFECTED WITH *E. TENELLA*

Pteridine % in Diet	Sulphadimidine % in Diet	Experiment 4		Experiment 5		Experiment 6		Experiment 7		Total		
		No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
0·01	0·05			12	1	11	1			23	2	9
0·02	0·025							12	5	12	5	42
0·02	0·05			12	0	11	0	12	0	35	0	0
0·03	0·025							12	6	12	6	50
0·03	0·05			12	0	11	1	12	1	35	2	6
0·01	—	12	4			11	9			23	13	57
0·02	—	12	8			11	11	12	10	35	29	83
0·03	—					11	10	12	10	23	20	87
0·04	—	12	9							12	9	75
0·05	—			12	11					12	11	92
0·06	—	12	11							12	11	92
0·08	—	12	4							12	4	33
0·1	—	12	7	12	11					24	18	75
0·12	—	12	4							12	4	33
0·15	—			12	5	11	5	12	6	35	16	46
0·2	—			12	8					12	8	67
—	0·025							12	10	12	10	83
—	0·05					11	7	12	7	23	14	61
—	0·08			12	5	11	7			23	12	52
—	0·1	12	0	12	1	11	3	12	1	47	5	11
—	0·12	12	0	12	1					24	1	4
—	0·14	12	1							12	1	8
—	—	12	8	12	10	11	10	12	11	47	39	83

TABLE IV

EFFECTS OF 2,4-DIAMINO-6,7-DI-ISOPROPYL- AND 2,4-DIAMINO-1′-PROPYLINDOLO(2′,3′:6,7)-PTERIDINE ON THE SULPHADIMIDINE TREATMENT OF CHICKS INFECTED WITH *E. NECATRIX*

Pteridine		Sulphadimidine % in Diet	Experiment 8		Experiment 9		Total		
Name	% in Diet		No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
Di-isopropyl	0·0025	0·025	10	6			10	6	60
	0·005	0·05	10	9			10	9	90
	0·01	0·1	10	2	10	1	20	3	15
	0·015	0·15			10	0	10	0	0
	0·02	0·2	10	0	10	0	20	0	0
	0·02	—	9	4			9	4	44
Propylindolo	0·0025	0·025	9	6			9	6	67
	0·005	0·05	9	6			9	6	67
	0·01	0·1			11	9	11	9	82
	0·015	0·15			11	3	11	3	27
	0·02	0·2			11	7	11	7	64
	0·005	—	9	7			9	7	78
	—	0·2	9	7	11	8	20	15	75
	—	—	10	6	11	8	21	14	67

TABLE V

EFFECTS OF 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE, SULPHADIMIDINE AND THEIR MIXTURES ON *E. NECATRIX* INFECTIONS OF CHICKS AND THE EFFECT OF *p*-AMINOBENZOIC ACID ON THEIR ACTION

% Drug in Diet			Experiment 10		Experiment 11		Experiment 12		Total		
Pteridine	Sulphadimidine	*p*-Aminobenzoic Acid	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	No. of Birds	No. of Deaths	% Mortality
0·01	0·1	—			9	1	8	1	17	2	12
0·01	0·15	—					8	0	8	0	0
0·01	0·2	—			9	1	8	0	17	1	6
0·015	0·1	—					8	0	8	0	0
0·015	0·15	—					8	0	8	0	0
0·015	0·2	—					8	0	8	0	0
0·02	0·1	—			9	1	8	1	17	2	12
0·02	0·15	—					8	0	8	0	0
0·02	0·2	—	8	0	9	0	8	0	25	0	0
—	1·0	0·01	8	7					8	7	88
0·02	0·2	0·01	8	4					8	4	50
0·01	—	—			9	4			9	4	44
0·02	—	—	8	8	9	8			17	16	94
0·04	—	—			9	5			9	5	56
—	1·0	—	8	2					8	2	25
—	0·2	—	8	8	9	5	8	4	25	17	68
—	—	—	7	7	8	6	9	5	24	18	75

2,4 - Diamino - 6,7 - di - isopropylpteridine with Sulphadimidine.—It will be shown below that the di-isopropylpteridine is more active than the propylindolopteridine as a sulphadimidine potentiator against *E. necatrix*. For this reason and because supplies were more readily avilable, the di-isopropylpteridine was selected for further study against *E. tenella*. The results of four experiments, involving 517 chickens, with this pteridine, sulphadimidine and their mixtures are summarized in Table III, from which it will be seen that each drug alone exerted some therapeutic action, though that of the pteridine was only just detectable at high dose levels. Mixtures of the two drugs were very much more effective, roughly equiactive treatments being: the pteridine alone, 0.15%, sulphadimidine alone, 0.08%, and a mixture of the pteridine 0.02% and sulphadimidine 0.025%.

Therapeutic Tests against E. necatrix

Comparison of the Di-isopropyl- and Propylindolo-pteridine as Potentiators of Sulphadimidine.—In two experiments, involving 180 chickens, 10% mixtures of each of the pteridines with sulphadimidine were compared. The results expressed in Table IV indicate clearly that the di-isopropylpteridine strongly potentiated sulphadimidine and that the propylindolopteridine potentiated it only slightly if at all. Sulphadimidine alone at 0.2% in the diet was inactive.

In a further three experiments, involving 224 chicks (Table V) the activities against *E. necatrix* infections of the di-isopropylpteridine, sulphadimidine and their mixtures were again compared and the results confirmed those shown in Table IV. Also as *p*-aminobenzoic acid is known to antagonize sulphonamides, we investigated in one of these experiments its effect on the therapeutic action of sulphadimidine and of a mixture of the di-isopropylpteridine and sulphadimidine. There were 7 deaths in 8 birds treated with sulphadimidine and *p*-aminobenzoic acid, and 2 in the corresponding group treated with sulphadimidine alone. There were 4 deaths in a group of 8 infected birds treated with *p*-aminobenzoic acid as well as the pteridine-sulphadimidine mixture, as compared with no deaths in the group treated with the pteridine-sulphadimidine mixture alone (Table V, Experiment 10).

Toxicity Studies in Chicks

Diets containing various concentrations of a mixture of 1 part of the di-isopropylpteridine with 9 parts of sulphadimidine were fed to groups

TABLE VI

DIETARY INTAKE OF 2,4-DIAMINO-6,7-DI-ISOPROPYLPTERIDINE AND SULPHADIMIDINE BY TWO-WEEK-OLD CHICKS

Groups of 10 chicks were tested at each dose level.

% in Diet		Amount of Drug Consumed in mg.	
Pteridine	Sulphadimidine	Pteridine	Sulphadimidine
0·005	0·05	3·47	34·7
0·01	0·1	7·35	73·5
0·02	0·2	11·4	114
0·04	0·4	21·4	214
0·08	0·8	56·0	560

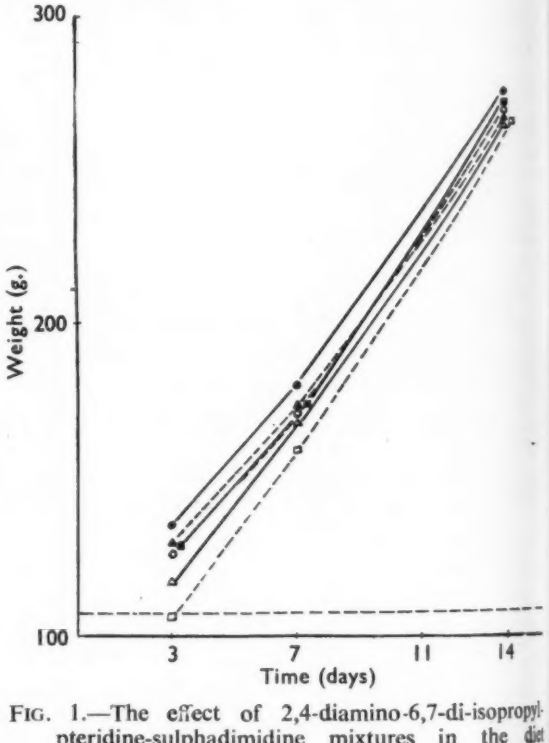

FIG. 1.—The effect of 2,4-diamino-6,7-di-isopropylpteridine-sulphadimidine mixtures in the diet on the weight gain of chicks during and after treatment. Treatment from Day 0 to Day 3. The horizontal line indicates the initial mean weight of all birds at Day 0 (107·8 g.). ●——● = Untreated control. ▲ — — — ▲ = Pteridine 0.005%, sulphadimidine 0.05%. ■ ——— ■ = Pteridine 0.01%, sulphadimidine 0.1%. O— — —O = Pteridine 0.02%, sulphadimidine 0.2% △ ——— △ = Pteridine 0.04%, sulphadimidine 0.4%, □— — —□ = Pteridine 0.08%, sulphadimidine 0.8%.

of 10 two-week-old chicks for 3 days. The amount of drug consumed was calculated and the body weight of the chicks was observed. The amount of drug consumed is given in Table VI and the weight gain in Fig. 1, which shows that drug treatment at high concentrations depressed weight gain and this gradually recovered after the withdrawal of treatment.

Discussion

In discussing the combined action of sulphadimidine and pteridines, the terminology described by Gaddum (1940) will be used. In this, the term "synergism" refers to any kind of joint action that is not antagonism, and "potentiation" refers to synergism that is greater than additive. From the experiments reported above it would be difficult to provide formal proof of potentiation by constructing a diagram of the type illustrated by Gaddum. However, a number of experiments strongly suggest that the pteridines potentiate sulphadimidine against both species of *Eimeria*. For *E. tenella* these experiments are Nos. 3, 5, 6, and for *E. necatrix* No. 11. In the other experiments, the mixtures generally show therapeutic action, but the absence of results at appropriate dose levels made it difficult to conclude that potentiation is present.

As strains of *E. necatrix* which do not respond to sulphonamide have been described (Horton-Smith and Long, 1959), the therapeutic action of the mixture of 2,4-diamino-6,7-di-isopropylpteridine and sulphonamide against this species might be of considerable importance. Mixtures of these drugs seem worthy of toxicological investigation with a view to field trial.

The brief experiment on toxicity of the di-isopropylpteridine-sulphonamide mixture in the diet of chicks reported above indicates some depression of growth, from which birds afterwards recovered, by a mixture fully active in the therapeutic tests. It is of interest that Collier, Hall and Waterhouse (1950) observed no potentiation by di-isopropylpteridine of sulphaguanidine toxicity in mice.

We are grateful to Allen and Hanburys Ltd. for the supply of pteridines used in this investigation.

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THE PHARMACOLOGY OF CHYMOTRYPSIN ADMINISTERED BY INHALATION

BY

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The ability of chymotrypsin to reach the limits of the bronchial tree has been studied in cats receiving the enzyme by inhalation as a very fine powder. For this purpose derivatives of chymotrypsin were used which had been labelled with a fluorescent molecule or with [^{131}I]. Quantitative measurements of the absorption and distribution of inhaled chymotrypsin- [^{131}I] revealed a rapid removal of radioactivity from the lungs over the first 24 hr. and corresponding excretion of labelled inorganic iodide in the urine. High levels of activity were not attained in the blood or thyroid. Subcutaneous administration of labelled enzyme led to more rapid accumulation of radioactivity in the blood and thyroid. Consideration of these and other results leads to the conclusion that, while some enzyme ascends the respiratory tract by ciliary movement of mucus, a substantial part is absorbed into the lungs and the [^{131}I] subsequently detached from it.

The changes in tidal air accompanying inhalation of labelled trypsin and chymotrypsin were followed in anaesthetized cats. Trypsin brings about a decrease in tidal air in distinctly lower doses than does chymotrypsin. Prior administration of mepyramine had an antagonistic effect, and it is suggested that the change in tidal air is essentially the result of bronchial spasm.

Where a drug is required to act locally within the lung it is advantageous to administer it by inhalation as a particulate cloud. However, one must ensure that an adequate proportion of the particles—by weight rather than by number—are of the requisite size for the site within the lungs which the powder is intended to reach and in which it has to be retained. Thus, one of the factors controlling the effectiveness of the drug is the homogeneity of the powder and particularly the low proportion of particles outside the desired range of sizes.

These considerations apply to the use of chymotrypsin as a mucolytic agent in the treatment of chronic bronchitis. Here the advantage is one of direct action on mucoprotein fibrils within the bronchial lumen. At present nothing is known of the subsequent disposal of the enzyme. We have reported here our attempts to ascertain whether the enzyme penetrates to all parts of the bronchial tree, what its subsequent distribution might be, and the degree of intolerance to the inhaled enzyme powder, in comparison with that shown to crystalline trypsin.

METHODS

Enzymes.—In the earlier stages crystalline chymotrypsin (Worthington) was employed, but the majority of the experiments were carried out with crystalline chymotrypsin as used in the therapeutic preparation "Lomudase." By the Anson procedure (Anson, 1938; Northrop, Kunitz, and Herriott, 1948) this was found to assay at 0.002 Anson units per mg. enzyme. The characteristics of this material are such that when it is dispersed in air with the clinical applicator ("Lomulizer") the resulting powder inhalation consists of particles of which about 80% by weight have diameters between 2 and 6 μ. Of the remainder about one-third by weight are in the range 1 to 2 μ and two-thirds 6 to 9 μ (Fowler, personal communication).

A check was also made on photomicrographs of particles collected on a microscope slide from a low density smoke produced by the "Lomulizer" in a chronic toxicity apparatus (to be described in a later publication). Under these conditions the majority of the particles were seen to be 3 to 7 μ in size. In each high power field there were one or two aggregates, each consisting of a chain of three or four particles.

The trypsin used was a commercial preparation (Armour) of the pure crystalline enzyme (0.011 Anson units per mg. enzyme). The particle size distribution of the inhaled trypsin powder was in the same range as that described for chymotrypsin.

Fluorescent labelling of chymotrypsinogen was carried out by a modification of the method of Hartley and Massey (1956). The final material, 1 - dimethylaminonaphthalene - 5 - sulphonylchymotrypsin, as used in the insufflation experiments was

sparingly soluble in water and contained, in different batches, 80 to 90% protein, 2.5 to 3.5 moles of label per mole of protein, and 76 to 79% of the proteolytic activity of the unlabelled enzyme. Its particle size distribution lay within the limits set for chymotrypsin.

Labelling of trypsin and chymotrypsin with [^{131}I] by the method of Veall, Pearson, and Handley (1955) involved the use of 2 mc. [^{131}I] per 200 mg. enzyme protein. The temperature was maintained below 5° throughout all the operations. To remove free iodine, the iodinated protein was precipitated with 4 volumes of acetone, the precipitate dissolved in water and dialysed against tap water. Samples were taken from the dialysis tubing and chromatographed, using butanol : pyridine : water (3 : 2 : 1.5) as a solvent. The chromatograms were scanned to determine the concentration of ionic iodine ; when the ionic iodine spot contained less than 1% of the total radioactivity, dialysis was discontinued. It is important not to dialyse over too long a period, since the enzymes diffuse through the Visking membrane. The preparation was then freeze-dried, the active product diluted with 4 times its weight of the original enzyme, and converted to a very fine powder having the same characteristics as those described for chymotrypsin. The resulting chymotrypsin and trypsin powders possessed specific activities of 76,000 and 43,500 counts/min./mg. and 94% and 92.5% respectively of the proteolytic activities of the crystalline enzymes. The radioactive enzymes contained less than 1% ionic iodine.

Determination of Metabolic Fractions.—For protein-bound [^{131}I] in plasma, urine, and alimentary tract washings, the protein was precipitated by the addition of an excess of 20% trichloracetic acid to a known volume of urine or plasma. The precipitate was removed by centrifugation, washed with 20% trichloracetic acid, and dissolved in 10 ml. N sodium hydroxide to determine the activity.

To determine the inorganic iodine, it was precipitated from the trichloracetic acid supernatant by the addition of an excess of a solution containing 20% silver nitrate and 10% nitric acid. The precipitate was suspended in water and its activity determined. Butanol-soluble iodine (thyroxine, diiodotyrosine, and inorganic iodine) was estimated by extracting 5 ml. of plasma or urine with 5 ml. *n*-butanol, and measuring the activity of the butanol layer. Since this was low, it was decided not to fractionate the extract into its component forms of iodine.

Insufflation Procedures.—All the enzymes were converted to very fine powders approximating as closely as possible to the chymotrypsin of "Lomudase." They were packed into cartridges and dispersed precisely as was done with chymotrypsin.

Indirect method A.—Cats under chloralose and urethane anaesthesia were connected by intratracheal cannula to the insufflation apparatus shown in Fig. 1. Here a measured volume of air (0.2 to 20 ml., according to the dose of powder required) was blown from a syringe through the dispersion unit B in which was placed a cartridge containing the powdered enzyme. The dense cloud of powder impinged on the baffle plate C, the purpose of which was to break up aggregates of powder, and passed into a large jar fitted with a magnetic stirrer D. The airborne suspension of powder was mixed with air entering the jar from a respiration pump at F and passed to the cat through tube G or to an excess volume recorder at E. The tracheal cannula had an outlet to I through the electro-magnetic clamp H which was closed on inspiration, thus making it possible to lead out of the laboratory all expiratory air containing radioactive powder.

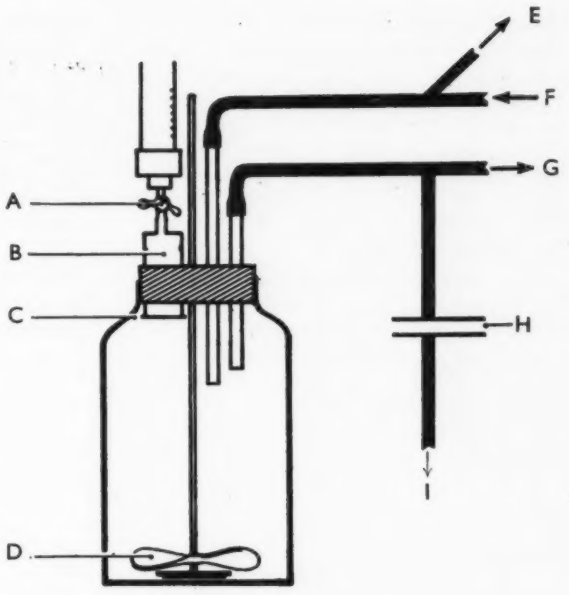

FIG. 1.—Diagram of apparatus used in the administration of powders by inhalation to anaesthetized cats by the indirect method A. A, Syringe with two-way tap; B, dispersion unit; C, baffle plate; D, stirrer (magnetic); E, tube to excess volume recorder; F, air inlet from respiration pump; G, tube to tracheal cannula; H, electro-magnetic clamp closed on inspiration; I, expiratory air outlet.

Changes in the distensibility of the cat lungs at a constant pressure of 9.5 cm. of water were measured by the overflow method of Konzett and Rossler (1940); blood pressure was simultaneously recorded from the carotid artery. The tidal air change was calibrated for each experiment in terms of the pressure, in cm. of water required to produce an equivalent excursion of the recorder.

Direct method B was used to attain higher lung concentrations for autoradiography and other radioisotope studies. The apparatus shown in Fig. 1 was modified by connecting the outlet of the dispersion unit B directly to the tube leading to G. A 50 ml.

syringe replaced the excess volume recorder at E. To insufflate powder, the respiration pump was stopped and, with the lungs of the cat momentarily deflated, clamps were applied to the tube leading from F and to the side-tube leading to H and I. Immediately air was expelled through the dispersion unit B and from the syringe at E into the lungs. By means of this syringe the lungs were deflated and inflated several times before the clamps were removed, and artificial respiration resumed with the pump at F.

Post-mortem Observations.—The cats were killed at varying times after insufflation of enzyme. The lungs and tracheae of animals which had received fluorescent labelled chymotrypsin were examined macroscopically under ultraviolet light. In some experiments frozen sections of the lung were studied by ultraviolet microscopy.

The lungs and tracheae of animals which had inhaled radioactive enzymes were used either for quantitative determination of the amount of enzyme present or for autoradiography. In carrying out radioassay of the labelled enzymes, the trachea was cut off at the carina and the trachea, main bronchi, and lungs separately homogenized in a known volume of 5% sodium hydroxide. The activity of the homogenate was determined in an EKCO N530 Scintillation Counter fitted with an annular type crystal. In some animals, determinations were also made of the radioactivity present in the liver, thyroid, stomach, small intestine, blood, and urine.

Autoradiographs were prepared by embedding the trachea and lungs in paraffin wax, cutting the blocks so as to obtain a flat, transverse section through the lung and trachea and placing each block in contact with an Ilford B *x*-ray film for 7 days.

Results

Experiments with Fluorescent Enzyme.—Untreated cat lungs exhibit a blue fluorescence in ultraviolet light. In cats killed immediately after insufflation of 3.2 mg. fluorescent chymotrypsin the terminal portion of the trachea, bronchi, and lung tissue showed a faint yellow fluorescence in ultraviolet light. In lung sections examined by ultraviolet microscopy (Lempert, 1944) the fluorescence was seen to emanate from discrete particles present throughout the lung right to the periphery, but was too weak to photograph in our apparatus. Moreover, attempts to use fluorescent counterstains resulted in quenching of the enzyme fluorescence. In Fig. 2 is shown a photomicrograph of one such lung section, for which we are indebted to Dr. A. Jarrett and Mr. T. Bligh of University College Hospital Medical School, London. It demonstrates that the inhaled powder has penetrated deeply into the lung, as far as the bronchioles; one bronchiole is shown whose lumen is lined by fluorescent enzyme.

When these experiments were repeated, but the cats killed 1 hr. after enzyme inhalation, no fluorescence was detected macroscopically in the smaller bronchi. Accordingly the procedure was modified by ligating the hilum of one lung after the insufflation of enzyme. One hour later the cat was killed and now, while no fluorescence was detected in the unligated lung, there was a uniform distribution of fluorescence in the bronchi and bronchioles of the ligated lung. In further experiments a polythene annulus was introduced into one main bronchus in order to prevent ciliary transfer of mucus past the ring while maintaining normal blood flow and aeration to the lung distal to it. The enzyme was then inhaled by the cat, which was killed 1 hr. later. No fluorescence was detectable in either lung, but an accumulation of fluorescent material was found to have taken place in the bronchial lumen immediately beyond the ring.

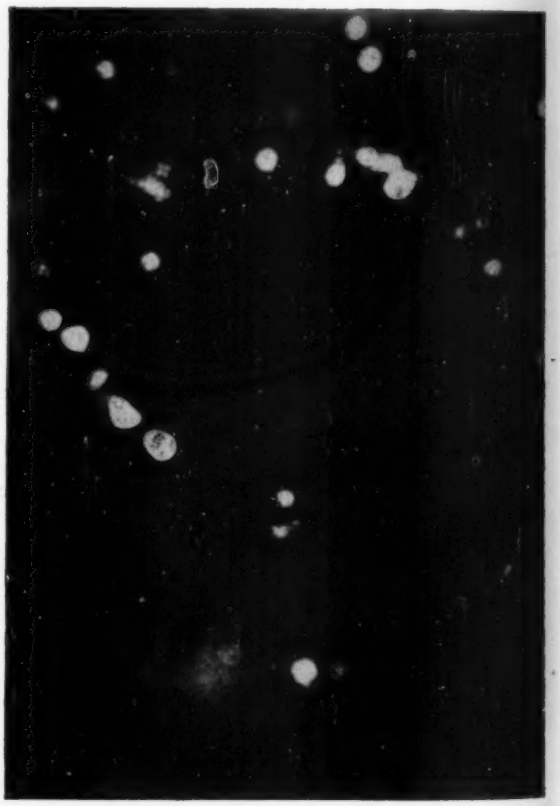

FIG. 2.—Ultraviolet photomicrograph of a section through cat lung following inhalation of fluorescent-labelled chymotrypsin, showing a bronchiole whose lumen is lined by fluorescent particles of enzyme. (Original magnification ×840.)

It was concluded from these experiments that the particulate enzyme, when administered by inhalation, was able to reach to the periphery of the lung but that it was rapidly cleared from the lung. In part at least, such clearance was taking place by the upflow of mucus in the bronchial tree. Attempts were then made to place these observations on a quantitative basis by fluorimetry of the labelled enzyme. However, the intrinsic fluorescence of the lung tissue precluded such measurements.

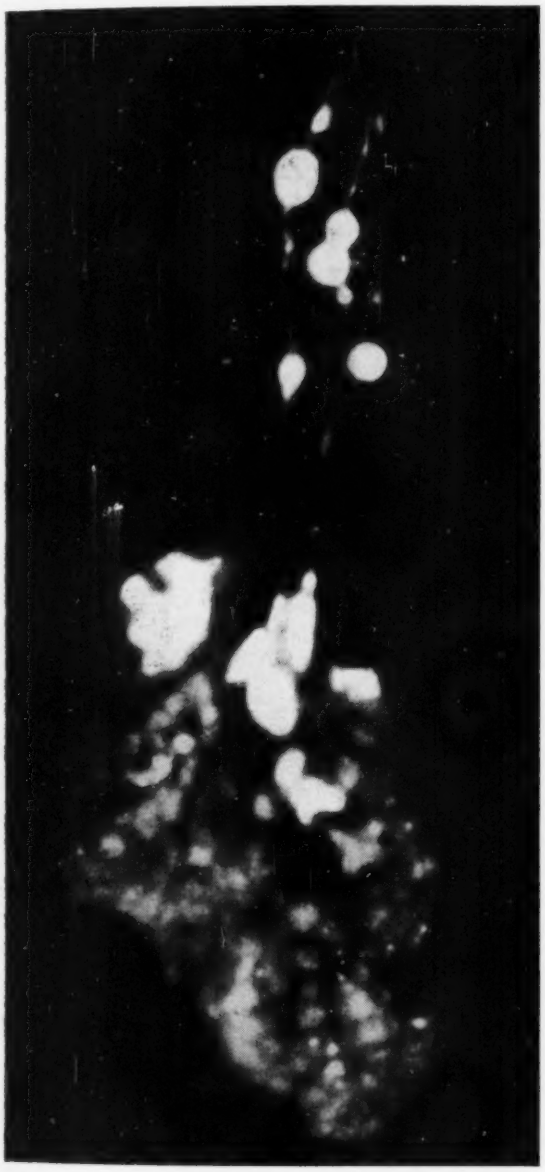

FIG. 3.—Autoradiograph of a section of the trachea, main bronchus and lung of a cat showing the penetration of inhaled chymotrypsin-[^{131}I]. The remarkable uniformity with which the radioactive material has been distributed throughout the lung is clearly demonstrated. Above is seen the heavy deposit, presumably of larger-sized particles, in the more proximal parts of the trachea.

Chymotrypsin Labelled with [^{131}I].—Preliminary experiments showed iodine-labelled chymotrypsin to be eminently suitable for distribution studies. Autoradiography was also possible since normal cat lung produced no artefact under the conditions used. An autoradiograph of a section through the trachea, main bronchus, and lung of a cat is shown in Fig. 3. The animal had been killed immediately after it had inhaled chymotrypsin-[^{131}I], of which 1.0 mg. was retained in the lungs. There is a uniform distribution of radioactive material throughout the lung, whereas in the more proximal part of the trachea a heavy deposit is present. It is possible that such tracheal sedimentation involves the larger particles.

Although there was considerable variation from one experiment to the next in the quantity of enzyme retained in the lungs, approximately 10% of this amount was always found in the trachea. In order to follow the changes in distribution of radioactivity with time, a series of experiments

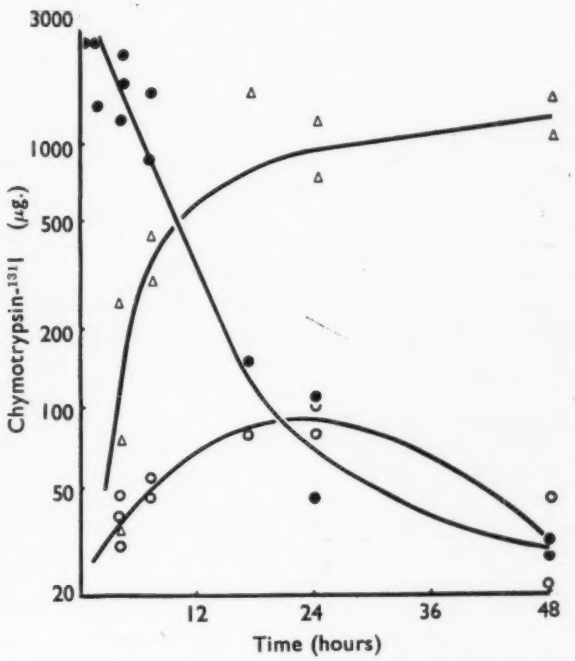

FIG. 4.—Distribution of radioactivity in lungs ●——●, liver O——O, and urine Δ——Δ of cats at various times following the inhalation of chymotrypsin-[^{131}I].

TABLE I

DISTRIBUTION OF RADIOACTIVITY IN TEN CATS FOLLOWING ADMINISTRATION OF INORGANIC [^{131}I] OR CHYMOTRYPSIN-[^{131}I]

The results are expressed in μg. chymotrypsin corresponding to the observed radioactivity, or as a percentage of the radioactive dose administered. In inhalation experiments the dose of chymotrypsin refers to the weight of powder expelled from the dispersion unit. The quantities in the total blood of the cat have been calculated on a basis of blood volume (ml.) = 52 × body weight (kg.).

Method of Administration				Time after Administration (hr.)	Results of Analysis (μg.)				
Material	Dose		Route		Thyroid	Lungs	Trachea	Whole Blood	Urinary Excretion (from Start)
	mg.	μc.							
Iodide ..		1·0	Subcutaneous	5·5	8·7%			4·4%	25·5%
,, ..		1·0	,,	5·5	6·3%			4·7%	
Chymotrypsin ..	3·0	1·0	,,	5·5	70 (2·3%)			214 (7·1%)	99 (3·3%)
,, ..	3·0	1·0	,,	5·5	359 (12·0%)			132 (4·4%)	267 (8·9%)
,, ..	28·8		Inhalation (Method A)	5·5	21	1,000	28	108	619
,, ..	30·8		,,	5·5	24	1,232	28	93	525
,, ..	17·6		,,	24	200	31	1	38	408
,, ..	42·0		,,	24	295	145	19	92	1,020
,, ..	14·3		Inhalation (Method B)	6·0		2,320	263	350	249
,, ..	13·2		,,	24		377	40	130	780

was carried out, with the results shown in Fig. 4. It is clear that activity is present in the liver and urine within 4 hr. after insufflation of the enzyme. A second dose of radioactive enzyme was absorbed very much like the first. A comparison was made of the behaviour of ionic [^{131}I] and chymotrypsin-[^{131}I] given subcutaneously and by insufflation. This study revealed a rapid uptake of [^{131}I] by the thyroid from both materials given subcutaneously. Equally, there was rapid excretion in the urine (Table I). When the labelled chymotrypsin is given by inhalation, thyroid uptake is slow, in parallel with the slow clearance from the lung (see also Fig. 4). Only at 24 hr. does appreciable accumulation take place in the thyroid; by this time over 90% of the enzyme has been cleared from the lungs. It is worth noting that over the period 6 to 24 hr. there is no increase in tracheal radioactivity, as might be expected if the labelled material had been swept up from the lung by ciliary action.

In three preliminary experiments using direct method B for insufflation, 9.8, 8.5, and 10.1 mg. of chymotrypsin-[^{131}I] were expelled from the dispersion unit. The weights of enzyme present in the lungs were 2.30, 2.67, and 2.44 mg. respectively. The mean pulmonary retention was 26.4%, and the results emphasize the heavy, variable, and unpredictable loss of material on its way to the lungs and by exhalation. It is thus impossible to administer accurately a predetermined dose in any experiment; hence it is essential to measure the weight of enzyme retained in the lungs and trachea in each experiment. In Table II are given two typical sets of data for the distribution of radioactive fractions in some tissues of the cat at 6 and 24 hr. after it had inhaled chymotrypsin-[^{131}I] (method B). In view of the appearance of radioactivity in the stomach washings, the experiments were repeated, with division of the oesophagus between ligatures prior to insufflation of the enzyme. Radioactivity still appeared in the stomach washings within 4 hr., and consisted largely of iodide ion, thus readily accounting for its secretion into the gastric juice.

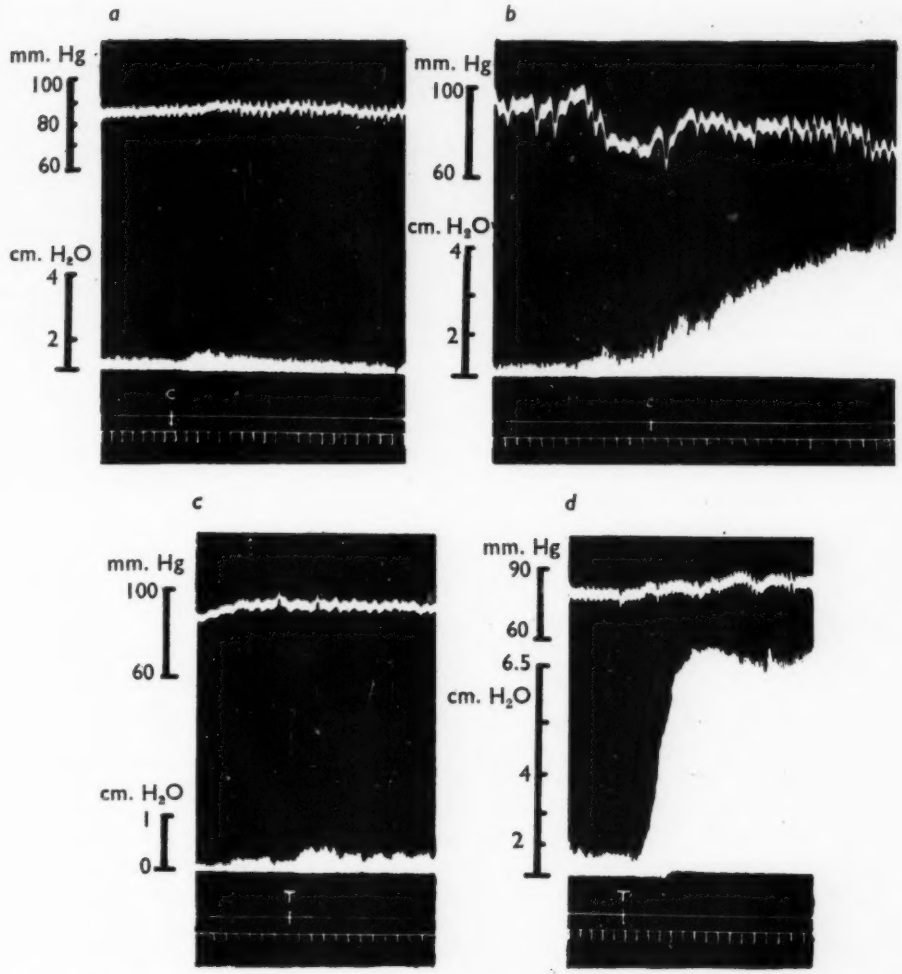

FIG. 5.—Records of tidal air and blood pressure following administration of trypsin and chymotrypsin to anaesthetized cats. The time scale is in min. The tidal air change was calibrated for each experiment in terms of the pressure, in cm. of water, required to produce an equivalent excursion of the recorder. (*a*) No significant alteration of tidal air after the insufflation of chymotrypsin-[^{131}I] at C. Radioassay of the trachea and lungs showed retention of 0.37 mg. enzyme. (*b*) Reduction of tidal air after the insufflation of chymotrypsin-[^{131}I] at C. Radioassay of the trachea and lungs showed retention of 1.09 mg. enzyme. (*c*) No alteration of tidal air after the insufflation of trypsin-[^{131}I] at T. Radioassay of the trachea and lungs showed retention of 0.023 mg. enzyme. (*d*) Reduction of tidal air after the insufflation of trypsin-[^{131}I] at T. Radioassay of the trachea and lungs showed retention of 0.08 mg. enzyme.

Tidal Air Changes.—Using anaesthetized cats as before, changes in tidal air were measured following the insufflation of [^{131}I]-labelled trypsin or chymotrypsin. The maximum dose was sought which could be administered without bringing about a reduction in tidal air. Whereas small amounts of trypsin decreased tidal air (Table III), preliminary tests showed that much greater doses of chymotrypsin could be given without eliciting such changes. Accordingly efforts were made to deposit several hundred micrograms of chymotrypsin within the lungs. From the results in Table III it is clear that smaller amounts of trypsin than of chymotrypsin are capable of inducing an appreciable reduction in tidal air. In Fig. 5 are shown tracings from 2 experiments in which the amounts of trypsin retained in the lungs were 23 and 80 μg. respectively. In the second experiment

there was a rapid decrease in tidal air (represented by an increased excursion of the lever measuring ventilation overflow), reaching its maximum in 5 min. Corresponding tracings with 370 and 1,090 μg. chymotrypsin are also shown in Fig. 5.

TABLE II

DISTRIBUTION OF RADIOACTIVE FRACTIONS IN SOME TISSUES OF THE CAT FOLLOWING INHALATION OF CHYMOTRYPSIN-[^{131}I]

The results are expressed in μg. chymotrypsin equivalent to the radioactivity observed. In the 6 and 24 hr. experiments 14.3 and 13.2 mg. respectively of chymotrypsin-[^{131}I] were expelled from the dispersion unit (method B). The total plasma volume (ml.) of the cat has been taken as 30 × body weight (kg.).

Specimen Analysed	Time after Administration (hr.)	Radioactive Fraction (μg.)			
		Inorganic Iodide	Protein-bound Iodine	Butanol-soluble Iodine	Total
Plasma ..	6	195·0	57·0	23·6	275·6
,, ..	24	—	—	—	99·5
Urine ..	6	236·0	0	12·7	248·7
,, ..	24	737·8	23·1	19·2	780·1
Stomach (washings)	6	202·2	—	—	—
Duodenum (washings)	6	42·5	—	—	—

TABLE III

CHANGES IN TIDAL AIR IN CATS FOLLOWING INHALATION OF TRYPSIN-[^{131}I] AND CHYMOTRYPSIN-[^{131}I]

Each result represents an experiment on one cat. Tidal air changes were measured by the method of Konzett and Rossler at a constant pressure of 9.5 cm. of water. The change in each experiment was calibrated in terms of the pressure, in cm. of water, required to produce an equivalent excursion of the recorder.

Decrease in Tidal Air (cm. Water)	Weight of Enzyme Present in Lungs and Trachea (μg.)	
	Trypsin	Chymotrypsin
0	5, 10, 23, 55	160, 370, 400, 480
1·0	5, 25	260, 530, 630
2·0	—	630
2·5	25	—
4·0	80, 320	1,090
6·5	80	—

The higher dose had a slow effect; about 13 min. elapsed before the maximum change was recorded. Repeated doses of either enzyme brought about no further change.

The carotid pressure was not affected by trypsin or chymotrypsin inhalation. It may be noted here that Werle, Kehl, and Koebke (1950) attributed the slight fall of blood pressure in dogs given crystalline trypsin to the presence of small amounts of adsorbed kallikrein.

The reduction in tidal air is most probably the result of bronchoconstriction, but its exact nature and mechanism have yet to be elucidated. It could be overcome in part by intravenous aminophylline (10 mg./kg.) or isoprenaline (0.5 to 2.0 μg./kg.) given by the same route. Prior administration of mepyramine (2 mg./kg. intravenously) in some cats completely abolished, and in others very substantially suppressed, the response to heavy doses of trypsin (195 to 300 μg. present in the lungs) or chymotrypsin (870 to 1,134 μg. present in the lungs). With mepyramine given only when the plateau of response to the enzyme had been reached, the same consistent effect was not seen.

DISCUSSION

Evidence of Lung Penetration.—The problem of ensuring pulmonary penetration of particulate drugs is primarily one of adequate dispersion of existing aggregates and prevention of formation of new ones. Since the objective was bronchiolar rather than alveolar retention of the powder, the low proportion by weight of particles below 2 μ in diameter was a decided advantage. On the other hand, the range selected as most suitable for human clinical use, and used in these experiments, is not necessarily the ideal one when the intention is to deposit the particles in the bronchioles of cats or other species of laboratory animals.

The evidence presented makes it clear that under the experimental conditions employed chymotrypsin does penetrate throughout the lungs. For technical reasons the precise localization and relative distribution of the enzyme particles in alveoli, alveolar ducts, various orders of bronchioles and bronchi could not be accurately determined. Ultraviolet fluorescence microscopy revealed a very generalized and singularly uniform distribution of the enzyme, but all attempts to counterstain the tissue quenched the weak fluorescence of the enzyme. Similarly [^{131}I], while it is a convenient label for quantitative studies, does not lend itself to high resolution in autoradiography.

Disposal of Inhaled Enzyme.—The speed with which the enzyme dissolves will to a great extent determine the relative parts played by ciliary clearance of enzyme-impregnated mucus and by direct absorption of chymotrypsin into the lung. The experiments with the relatively insoluble fluorescent enzyme suggested that some of the material was being cleared by centripetal movement up the bronchi—and very rapidly too, for within 1 hr. no fluorescence was seen in the lung macroscopically unless its activities had been suspended by a ligature at the hilum. The use of an intrabronchial polythene ring led to an accumulation of fluorescent material in the bronchus on the side distal to the ring, again pointing to ciliary clearance. (Small amounts of exogenous fluorescent material were no doubt still present in the lung but were impossible to detect owing to the intrinsic fluorescence.)

On the other hand, the clearance and distribution data with the readily soluble chymotrypsin-[^{131}I] are more precise. These results were relatively unaltered when the oesophagus was divided between ligatures prior to insufflation of the enzyme, so they cannot be attributed to ciliary clearance, swallowing and gastro-intestinal absorption. For at least 12 hr. after insufflation there is an exponential rate of disappearance of radioactivity from the lung, continuing at a low level over the next 36 hr. The possibility that liberation of [^{131}I] takes place within the bronchial lumen cannot be entirely excluded, but *in vitro* experiments with cat mucus make it clear that such a mechanism can contribute little to the overall picture. If the conclusions of Barnes and Trueta (1941) regarding subcutaneous absorption are applicable to the lung, chymotrypsin (composed mainly of α-chymotrypsin dimer with molecular weight of about 42,900) should be absorbed by the lymphatic route ; but it is conceivable that its proteolytic activity would bring about an increase in permeability to facilitate its own absorption, as has been suggested for trypsin (Martin, Bogner, and Edelman, 1957).

The rapidity of uptake from the lung and excretion into the urine suggests an artefact brought about by splitting off the radioactive iodine, which would then be readily removed. On the other hand, similar results have been obtained by us with water-soluble carboxylic acids labelled with [^{14}C] and administered in the same way (unpublished observations). The extent of liberation of inorganic iodine from the labelled enzyme was measured by incubating cat lung homogenate with chymotrypsin [^{131}I] for periods of up to 24 hr. at 37°. The supernatant after precipitation of the proteins contained only up to 18% of the total radioactivity present ; of this, one-half was inorganic iodide demonstrable by chromatography (unpublished observations). It is safe to conclude that no rapid splitting of the label from the enzyme takes place. This conclusion is borne out by the low activity appearing in the thyroid after the first 5.5 hr.

The subsequent disposal of the radioactive material, whatever it may be, appears to follow similar lines to those after subcutaneous injection —whether of chymotrypsin-[^{131}I] in cats or trypsin-[^{131}I] in rats (Bogner *et al.*, 1959). Unfortunately no data for urine are provided by these authors. On the other hand, the subcutaneous route does lead to a more rapid accumulation of activity in the blood and in the thyroid gland.

The question also arises of the secretion of free radioiodine into the lungs and especially the mucous glands of the trachea and bronchi. Eichler and Schütterle (1958) claimed that preferential concentration of ^{131}I occurs in the trachea of the guinea-pig and rat within 2 hr. after intraperitoneal administration. Subsequently (Eichler and Sebening, 1959) the results in rats were not confirmed, but in the cat trachea an enrichment of radioiodine of 1.25 over the plasma value was observed. Following inhalation of chymotrypsin-[^{131}I] under the conditions described here there was no evidence to suggest preferential uptake of [^{131}I] by the trachea whereas secretion into the stomach was readily demonstrable at 4 hr. Pulmonary secretion of the label is unlikely to be playing a significant part in its disposal during the first 12 to 24 hr.

Tidal Air Changes.—Barer and Nusser (1953) and Konzett (1956) have emphasized the difficulty of attributing a decrease in tidal air to bronchoconstriction rather than pulmonary congestion. With histamine or 5-hydroxytryptamine the response is immediate and certainly points to a direct action on the bronchial musculature (Konzett, 1956). The tidal air change elicited by trypsin is of the same type, whereas chymotrypsin, in high doses, produces its effect gradually, reaching a plateau only after several minutes. Despite the different character of these changes in the anaesthetized cat, both were largely or completely abolished by pretreatment with mepyramine.

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PHARMACOLOGICAL STUDY OF A NEW ANTIBIOTIC OF BACILLARY ORIGIN

BY

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The purified extract of a new bacterial mutant, which exhibits antibiotic activity against paramecia and amoebae, was tested on various smooth muscle preparations. Spontaneous activity as well as the response to a variety of smooth muscle stimulants was diminished or completely suppressed. Intravenous injection of the extract lowered the blood pressure of the anaesthetized cat or dog. These properties may explain the beneficial effect of the extract on the intestinal symptoms of human amoebiasis, before eradication of the parasites.

In 1946, Reitler and Boxer isolated a new bacterial strain from the mesenteric lymph glands of a patient who succumbed to ileus with multiple intussusceptions of the jejunum. This strain grew in the form of short, Gram-negative rods, which produced spores abundantly. It also showed interesting antibiotic properties and exhibited a marked tendency to mutate under a variety of experimental conditions. Systematic selection led to mutants, which possessed not only antibacterial activity but produced also antiprotozoal substances in increasing quantities. One of these mutants, labelled RB-103, distinguished itself by its filamentous growth, its weak tendency to sporulation and its regular production of material, active against paramecia and even to a higher degree against amoebae (Reitler and Berner, 1960).

Purified extracts from the sub-strain RB-103 have been tried on several hundred patients suffering from acute or chronic infection with *Entamoeba histolytica*. Marked improvement of the intestinal symptoms was usually noted after a few days of treatment, but examination of the stools at this stage still revealed the presence of numerous amoebae and cysts. Analysis of the faeces became negative only after 2 to 3 weeks of continuous administration of the antibiotic. Such observations led to the idea that, in addition to the antibiotic substances, the bacterial extract may contain principles that act on the smooth muscle of the intestine and thus could be responsible for the pronounced change of the clinical picture long before elimination of the parasites (Reitler, 1950). The experiments described in this paper were designed to test this hypothesis and to investigate in general the pharmacodynamic properties of the antibiotic of the new strain.

METHODS

Antibiotic Material.—A sterile extract of the mutant RB-103, representing the last stage in the purification process, immediately before adsorption on to a solid carrier, was put at our disposal by Messrs. Hillel Remedies Inc., Haifa. The antibiotic activity of the preparation was tested against *Paramecium caudatum*, which is first immobilized and, during 24 hr., undergoes lysis. The extract used caused complete lysis at a dilution of 1 in 200. For the sake of brevity, we shall use in this paper the commercial name "Colisan" for the antibiotic preparation.

Isolated Organs.—Smooth muscle organs were suspended in an organ bath at 38°, containing aerated Locke solution. When the response to graded doses of a stimulant was irregular (for example, with the ileum of the guinea-pig), it was advantageous to store the organ in the refrigerator overnight. In the experiments described, figures about reagents refer to the final concentration of the substance in Locke solution.

Whole Animals.—Cats and dogs were anaesthetized with intravenous pentobarbitone, after induction with ether. Blood pressure was recorded from the left carotid artery, using an Hg manometer, and respiration by a membrane manometer, connected to the side arm of the tracheal cannula. Contractions of the left gastrocnemius were produced by stimulation of the sciatic nerve, placed on shielded silver electrodes, and registered by attaching the muscle to an isometric lever.

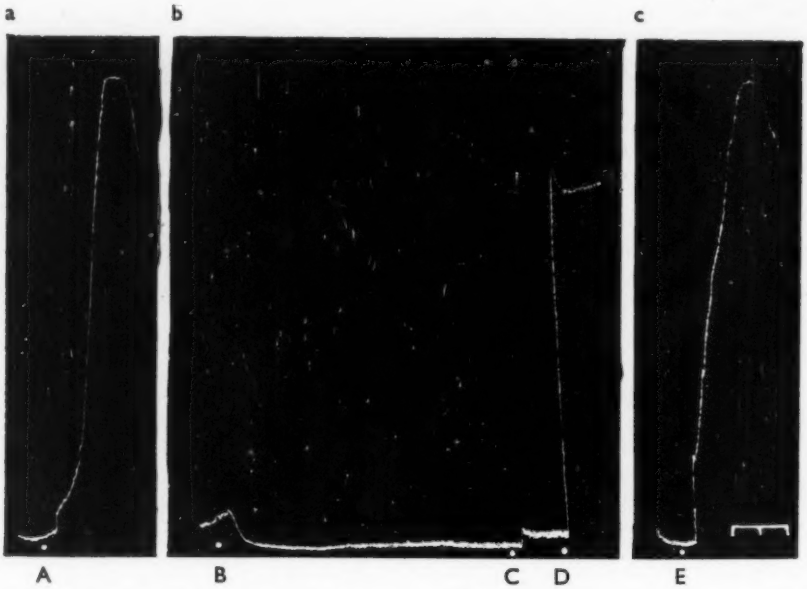

FIG. 1.—Antagonism of acetylcholine and colisan on the isolated guinea-pig ileum. A, Acetylcholine, 0.004 μg./ml. B, Colisan, 1 in 100 final dilution. C, Without washing, addition of acetylcholine, 0.004 μg./ml. D, Without washing, addition of acetylcholine, 0.16 μg./ml. Between D and E, repeated washings. E, Acetylcholine 0.004 μg./ml. Time marker, 1 min.

RESULTS

Effect of Colisan on the Guinea-pig Ileum.—An intestinal loop, incubated with colisan, showed a progressively diminishing response to acetylcholine. After 5 to 7 min. complete inhibition was obtained (Fig. 1). This effect was reversible; after repeated washing, the loop returned to its original sensitivity. The effect of colisan is competitive with the stimulant drug, because concentrations of acetylcholine, 10 to 50 times higher than the one used before inhibition, provoked a response even after 10 min. incubation with the bacterial extract.

The inhibitory effect can also be demonstrated in the reverse manner. Addition of colisan at the height of the contraction, due to stimulation by acetylcholine, led to immediate relaxation of the ileum (Fig. 2). This reverse reaction is distinguished from the effect of previous incubation not only by the time factor but also by the lower dose of colisan needed for complete relaxation (about 1/3 to 1/5 of the dose required in the incubation method).

The reverse method, because of its immediate effect, lends itself to a quantitative estimation of the activity of colisan. Increasing doses of the drug produce an increasing spasmolytic action (Fig. 3). By plotting log colisan concentration against the percentage decrease of the amplitude of the contraction induced by acetylcholine, a straight line is obtained (Fig. 4).

Effect of Colisan on the Guinea-pig Colon.—Analogous observations were made on the isolated colon. The spontaneous peristaltic movements of the organ, as well as the contractions produced by various stimulants, were abolished by colisan, which clearly exerts a threefold action (Fig. 5): (*a*) It lowers the tonus of the smooth muscle; (*b*) it diminishes the amplitude of the contractions; and (*c*) it reduces the frequency of the peristaltic movements.

Non-specificity of the Antagonism Between Colisan and Stimulants of Intestinal Smooth Muscle.—Colisan exerts its effect against any one of the following stimulants: acetylcholine, histamine, 5-hydroxytryptamine, nicotine, γ-amino-butyric acid and barium chloride. When equiactive doses of stimulants were compared, approximately the same concentration of colisan was required to counteract their effect. The broad antispasmodic spectrum demonstrates the non-specificity of colisan, compared with the action of other known antagonists of smooth muscle stimulants. Thus, a dose of atropine sufficient to abolish the response to acetylcholine will

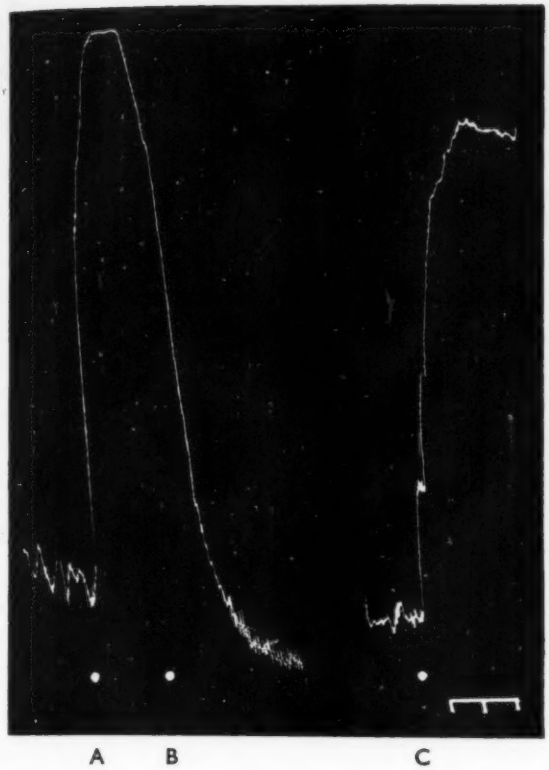

FIG. 2.—Effect of colisan, added at the height of a contraction of the guinea-pig ileum caused by acetylcholine. A, Acetylcholine, 0.02 μg./ml. B, Without washing, addition of colisan, 1 in 50 (final dilution). Between B and C, repeated washings. C, Acetylcholine, 0.02 μg./ml.

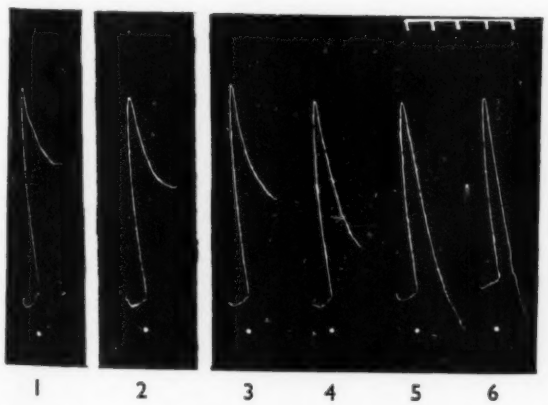

FIG. 3.—Graded effect of colisan on the contractions of the guinea-pig ileum, induced by 0.02 μg./ml. of acetylcholine. 1, Control. 2, Colisan 1 in 200. 3, 1 in 165. 4, 1 in 145. 5, 1 in 125. 6, 1 in 110. Note that 5 already produced maximal relaxation, while 6 produced this effect within a shorter time.

suppress only partially the response to histamine, 5-hydroxytryptamine, nicotine, or barium chloride. A given dose of the antihistamine diphenhydramine, which eliminates completely the contractions of the ileum induced by histamine or nicotine, is only partially effective against acetylcholine or 5-hydroxytryptamine. Florey's factor I shows specific antagonistic activity against acetylcholine and nicotine, but does not influence the response to 5-hydroxytryptamine (Florey and McLennan, 1959). Finally, botulinum toxin abolishes the contraction caused by nicotine, but does not interfere with the effect of acetylcholine or histamine (Ambache and Lessin, 1955).

Effect of Colisan on Other Smooth Muscle Organs.—The above experiments show that the relaxing effect of colisan is independent of the

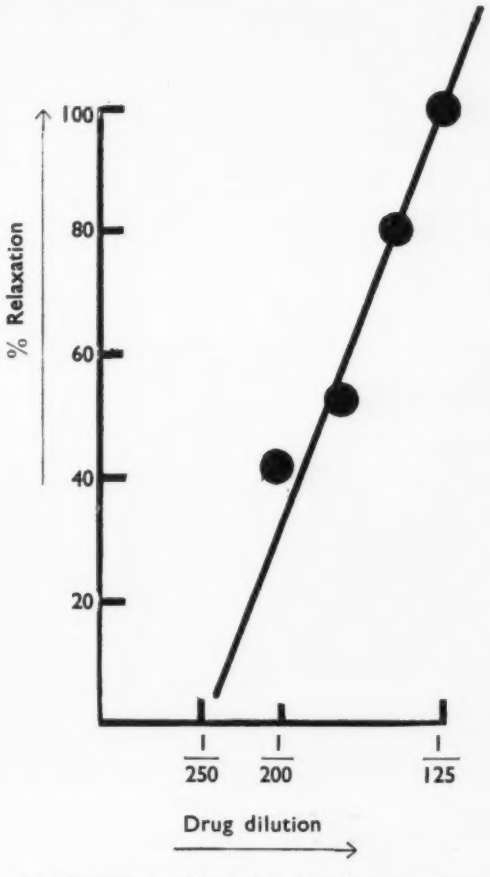

FIG. 4.—Percentage relaxation of guinea-pig ileum as function of log drug concentration. Taken from the data of Fig. 3. Ordinate,

$$\frac{\text{amplitude of descending limb of trace} \times 100}{\text{maximal amplitude of ascending limb}}$$

specific transmitter mechanism which operates in a given organ. This view is further supported by the observation that all smooth muscle organs tested showed essentially the same response to colisan as does the intestine. A survey of the organs examined and the agents used as most effective stimulants in each case is given in Table I, which also shows the species independence of the drug effect. For example, the non-pregnant uterus of the guinea-pig, which responds best to acetylcholine and histamine, and the uterus of the rat in the third week of pregnancy, which contracts after acetylcholine, 5-hydroxytryptamine (Fig. 6), and oxytocin, were both completely inhibited by colisan.

TABLE I

SMOOTH MUSCLE ORGANS TESTED FOR THE ANTAGONISTIC EFFECT OF COLISAN

The tests with γ-aminobutyric acid were carried out according to Hobbiger (1958).

Species	Organ	Stimulants Used
Guinea-pig	Ileum	Acetylcholine, histamine, 5 - hydroxytryptamine, nicotine, γ-aminobutyric acid, barium chloride
,,	Colon	Acetylcholine, histamine, 5 - hydroxytryptamine, nicotine, barium chloride
,,	Uterus (non-pregnant)	Acetylcholine, histamine, barium chloride
,,	Ureter	Histamine, nicotine, barium chloride
,,	Bladder	Histamine, barium chloride
Rat ..	Uterus (third week of pregnancy)	Acetylcholine, 5-hydroxytryptamine, oxytocin, barium chloride
,, ..	Stomach fundus	5-Hydroxytryptamine

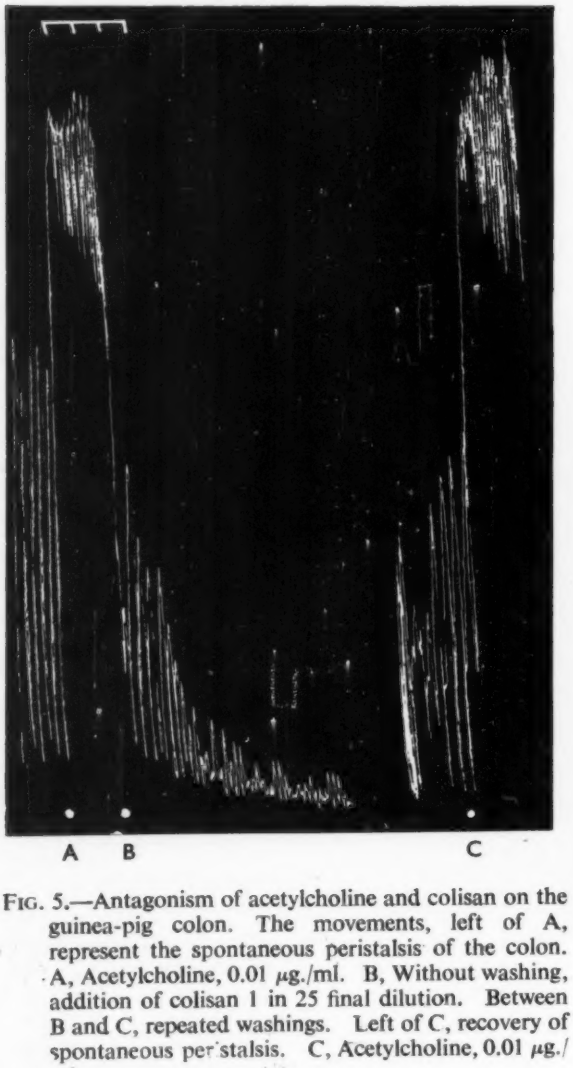

FIG. 5.—Antagonism of acetylcholine and colisan on the guinea-pig colon. The movements, left of A, represent the spontaneous peristalsis of the colon. A, Acetylcholine, 0.01 μg./ml. B, Without washing, addition of colisan 1 in 25 final dilution. Between B and C, repeated washings. Left of C, recovery of spontaneous peristalsis. C, Acetylcholine, 0.01 μg./ml.

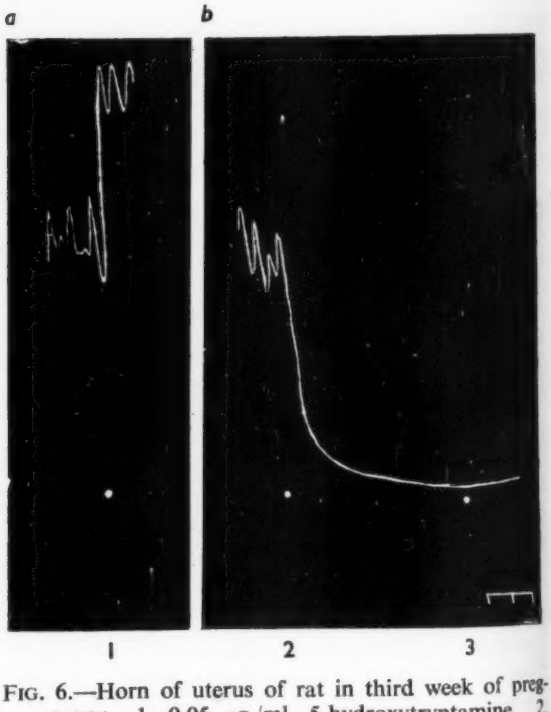

FIG. 6.—Horn of uterus of rat in third week of pregnancy. 1, 0.05 μg./ml. 5-hydroxytryptamine. 2, Colisan 1 in 100. After 7 min. incubation, addition of 0.05 μg./ml. 5-hydroxytryptamine at 3.

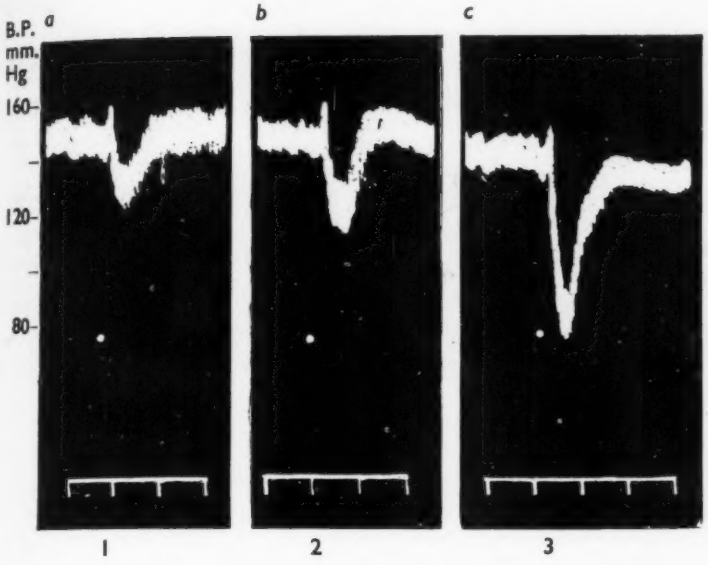

FIG. 7.—Effect of intravenous injections of colisan on the blood pressure of the dog under nembutal anaesthesia. 1, 0.5 ml. colisan. 2, 1.0 ml. colisan. 3, 2.0 ml. colisan.

Effect of Colisan on Striated Muscle.—Intravenous or intra-arterial injection of colisan into the cat had no effect on the amplitude of the contractions of the gastrocnemius, stimulated indirectly. Similar results were obtained with the phrenic-diaphragm preparation of Bülbring (1946).

Effect of Colisan on the Blood Pressure.—The response of the smooth muscle of the arterial wall was tested by measuring the blood pressure in the intact dog or cat. As shown in Fig. 7, after intravenous injection of colisan, the blood pressure fell immediately and returned within 1 to 2 min. to its original level. However, thereafter the blood pressure fell progressively to very low levels. Infusion of adrenaline raised the pressure again and restored the hypotensive response of the animal to colisan.

DISCUSSION

The extract from the bacterial mutant RB-103 contains a smooth muscle relaxant with a spectrum of activity much broader than that of other known antagonists to chemical stimulation, whether of animal, plant, or bacterial origin. The inhibitory efficiency of colisan against a whole array of smooth muscle stimulants, independent of their specific point of attack along the neuronal system or at the effector cell itself, supports the view that the drug acts directly on the muscle cell. The important fact should be stressed that, after incubation with colisan, any of the stimulating drugs tested can provoke contraction, if doses 10 to 50 times higher than the original ones are applied. Apparently, the antagonistic effect takes place at the contractile substance itself, a view supported by the observation that equal concentrations of colisan were required to neutralize the effect of equiactive doses of different stimulants.

The peculiar character of colisan is also revealed by comparison with other antagonists against smooth muscle stimulants. Florey (1954) isolated from brain extracts Factor I, which inhibits movements of the gut caused by acetylcholine or nicotine, but has no effect on the contraction provoked by 5-hydroxytryptamine (Florey and McLennan, 1959). In addition, these authors noted that about 25% of their biological preparations were insensitive to the brain extract. These properties distinguish Factor I from colisan, in view of the broad antagonistic spectrum of the latter (see Table I) as does the fact that its solutions, if active against one specific organ, are effective against all organs of the same or different species as far as has been tested.

The point of attack of colisan is also clearly demarcated from that of the botulinum toxins (Ambache, 1951). The latter abolishes contractions caused by nicotine, but does not impair the effect of acetylcholine, thus suggesting the neuronal parts of the preparation, that is, the synapse of the outer ganglion cells, the postganglionic neurons in the wall of the gut, or the nerve terminals at the muscle surface as locus of action. Furthermore, after about 10 min. incubation, the toxin cannot be washed out any more, whereas the effect of colisan is completely reversible by washing.

The situation is different with respect to staphylococcal β-toxin, which suppresses completely the spontaneous peristalsis of the isolated rabbit intestine as well as its response to acetylcholine (Anderson, James, and Marks, 1954; Kelsey and Hobbs, 1954). However, a comprehensive study of the localization of this antagonistic action has not been carried out. Therefore, it cannot be decided at present whether the toxin belongs to the same group as the active principle present in the extract of the mutant RB-103.

The present experiments support the hypothesis that the beneficial effect of colisan on human amoebiasis is due, at least in part, to the presence of a smooth muscle relaxant, which reduces the motility of the gut and thus alleviates the intestinal symptoms some time before the antiprotozoal effect becomes apparent. The pharmacodynamic observations with colisan naturally raise the question whether the antispasmodic principle of the bacterial extract is a separate entity or is identical with the antibiotic present in colisan. Experiments pertinent to this question will be reported in due course.

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THE PHARMACOLOGICAL ACTIONS OF 3,4-DIHYDROXYPHENYL-α-METHYLALANINE (α-METHYLDOPA), AN INHIBITOR OF 5-HYDROXYTRYPTOPHAN DECARBOXYLASE

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α-Methyldopa (3,4-dihydroxyphenyl-α-methylalanine) is an inhibitor of 5-hydroxytryptophan decarboxylase which is effective *in vitro* and *in vivo*. The inhibition is complex and shows coenzyme reversal. Evidence is presented that it acts by coenzyme inactivation. Its administration to mice reduced brain 5-hydroxytryptamine but had no effect on noradrenaline. After repeated doses, intestinal 5-hydroxytryptamine concentrations were also reduced. Co-ordinated activity was diminished coincidently with the enzyme inhibition and the reduction in brain 5-hydroxytryptamine. Rectal temperatures were reduced and the possibility that this resulted from inactivity is discussed. Treated animals showed miosis and narrowing of the palpebral fissures. A comparison is drawn between the actions of α-methyldopa and reserpine. The present results support the idea that the sedative effects of both drugs are due to the fall in brain 5-hydroxytryptamine concentration that they produce.

The endogenous formation of 5-hydroxytryptamine in body tissues depends on the activity of the enzyme 5-hydroxytryptophan decarboxylase (Clark, Weissbach and Udenfriend, 1954). This enzyme is widely distributed in the body and is found with a few exceptions wherever 5-hydroxytryptamine itself is present (Gaddum and Giarman, 1956). 3,4-Dihydroxyphenyl-α-methylalanine (α-methyldopa), an inhibitor of dopa decarboxylase described by Sourkes (1954), is effective also against 5-hydroxytryptophan decarboxylase *in vitro* and *in vivo* (Westermann, Balzer and Knell, 1958).

This paper concerns the action of α-methyldopa on 5-hydroxytryptophan decarboxylase *in vitro* and *in vivo*, and the pharmacological effects of its administration to mice and other animals. Results of preliminary experiments have been reported to the Physiological Society (Smith, 1959).

METHODS

Estimation of Decarboxylase.—Tissues were homogenized cold in physiological saline and were stored, when necessary, for up to 2 hr. in the refrigerator. Aliquots containing 250 mg. of tissue were incubated in 25 ml. conical flasks at 37° and shaken. The reaction mixture in the flasks was made up as follows:

1. Tissue homogenate	1 ml.
2. 0.5M phosphate buffer (*p*H 8.0) ...	2 ml.
3. Additions	1 ml.
4. DL 5-hydroxytryptophan 1.1 mg. (final concentration =1 μmole/ml.)	1 ml.

The mixture always contained iproniazid 0.89 mg. (final concentration 1 μmole/ml.) to inhibit amine oxidase. The substrate, 5-hydroxytryptophan, was added last after preincubation of the mixture for 15 min. 5-Hydroxytryptamine was assayed in the reaction mixture immediately after the addition of the substrate and again 30 min. later. Preliminary experiments showed that the rate of 5-hydroxytryptamine production was linear up to 1 hr.

Manometric Estimation of Decarboxylase.—In 1 experiment, 5-hydroxytryptophan decarboxylase was prepared from guinea-pig kidney by the method of Clark *et al.* (1954) as far as stage 3. CO_2 evolution was measured manometrically in Warburg flasks. The reaction mixture was made up as follows:

1. Enzyme preparation	2.0 ml.
2. 0.2M phosphate buffer (*p*H 8.0)	0.5 ml.
3. Pyridoxal 5-phosphate 30 μg. ...	0.1 ml.
4. Substrate (in side-arm 1) ...	0.2 ml.
5. 4N sulphuric acid (in side-arm 2)	0.2 ml.

The incubation periods were 20 or 40 min. The procedure was otherwise as described by Clark *et al.* (1954).

Assay of 5-Hydroxytryptamine. — Aliquots of incubation mixtures and of tissue homogenates were assayed fluorimetrically by the method of Bogdanski, Pletscher, Brodie, and Udenfriend (1956). Of the substances used in the experiments, α-methyldopa and pyridoxal 5-phosphate were found not to interfere with the fluorescence of 5-hydroxytryptamine. Iproniazid in the concentration used in the incubation mixtures quenched fluorescence, and a correction of +15% was found to compensate for the difference. This correction was applied when appropriate.

Assay of Noradrenaline.—The pressor action on the pithed rat (Shipley and Tilden, 1947) was employed. The extraction procedure used for tissues was as follows: brains were homogenized in 3 volumes (w/v) 0.02N hydrochloric acid, placed in deep freeze overnight and centrifuged the following day. The clear supernatant (*p*H 5.0) was injected directly into the test rats and assayed against noradrenaline. Although such a preparation probably contains other pressor and interfering substances, it seems unlikely that they interfere with the assay. Dopamine, which is present in the brain in about equal concentration, has only 1/100 of the pressor activity, and adrenaline, which is present in much lower concentration, has less activity than noradrenaline. Similar quantities of 5-hydroxytryptamine have a slight pressor activity on the preparation.

Mouse Activity.—The method of Dews (1953) was used, employing groups of 5 mice. This method appears to depend to some extent on the interest that the test animals display in their surroundings, and the activity counts diminish if animals are tested too frequently. In the experiments described no group was tested more than once in 48 hr., an arbitrary time chosen for lack of interference, and control groups were always tested at the same time as treated groups. Where the influence of α-methyldopa on activity has been followed against time, the results have been obtained with different groups over a period of many weeks.

Mouse Rectal Temperature.—This was measured with a small thermistor, inserted 2 cm. into the rectum, and supplied with current from a hearing-aid battery. The resistance of the thermistor was measured on a galvanometer, the full range of which was calibrated from 30 to 40°. Ambient temperatures varied from 22.6 to 25.3°.

Intestinal Passage of a Meal.—This was done by a modification of the method of Brittain and Collier (1958). The test meal given intragastrically consisted of 0.4 ml. of aqueous carmine solution 1%, instead of charcoal. This modification was applied so that an estimate could be made of the extent of gastric emptying during the test period. The animals were killed after 20 min., the stomachs removed, extracted with 0.1 N sodium hydroxide, filtered, and the carmine estimated colorimetrically against a standard curve using a green filter (Ilford 404). The percentage of the small intestine traversed by the meal was measured as described by the above authors.

Results

In Vitro *Experiments*

Action of α-Methyldopa on Mouse Brain 5-Hydroxytryptophan Decarboxylase. — The experiments confirmed that α-methyldopa is an effective inhibitor, 0.1 μmole/ml. producing 100% inhibition. The results are shown in Fig. 1. In the presence of added pyridoxal 5-phosphate, the natural coenzyme, 0.008 μmole/ml., it was much less effective and 10 μmole/ml. was now needed for complete inhibition. By interpolation, the doses needed to produce 50% inhibition were

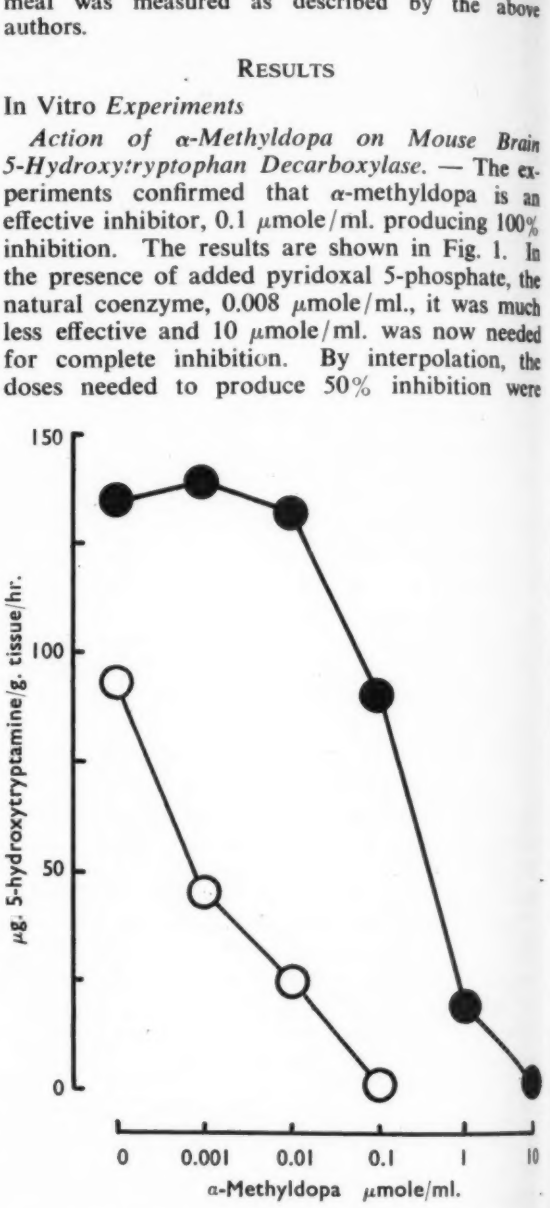

FIG. 1.—Mouse whole brain homogenate 5-hydroxytryptophan decarboxylase *in vitro*. Action of α-methyldopa. 5-Hydroxytryptophan and iproniazid concentrations each 1 μmole/ml. Ordinates: μg. 5-hydroxytryptamine formed/g. tissue/hr. Abscissae: α-methyldopa concentration in μmole/ml. incubation mixture. ●——● with added pyridoxal 5-phosphate 0.008 μmole/ml. O——O without addition.

0.0012 and 0.20 μmole/ml. respectively. In the absence of inhibitor, the addition of coenzyme increased the rate of decarboxylation from 87 to 135 μg. 5-hydroxytryptamine formed/g. tissue/hr. Further addition of coenzyme up to a concentration of 1 μmole/ml. had no effect.

Interaction of Substrate Concentration, Coenzyme Concentration and α-Methyldopa on Mouse Brain Decarboxylase.—Homogenates were incubated with varying concentrations of 5-hydroxytryptophan and pyridoxal 5-phosphate in the presence of a fixed concentration of α-methyldopa. The results are shown in Fig. 2. Increasing the concentration of coenzyme reduced the inhibition at any substrate level. Increasing the concentration of substrate also reduced the inhibition at any coenzyme level. The reciprocals of substrate concentrations (S) and of enzyme activities (V) were plotted graphically according to the method of Lineweaver and Burk (1934). Fig. 3 shows the results obtained at a coenzyme concentration of 1 μmole/ml. Regression lines for 1/V against 1/S at all coenzyme concentrations were calculated and the theoretical $1/V_{max}$ found in each case. The figures obtained are given in Table I. At low coenzyme concentrations

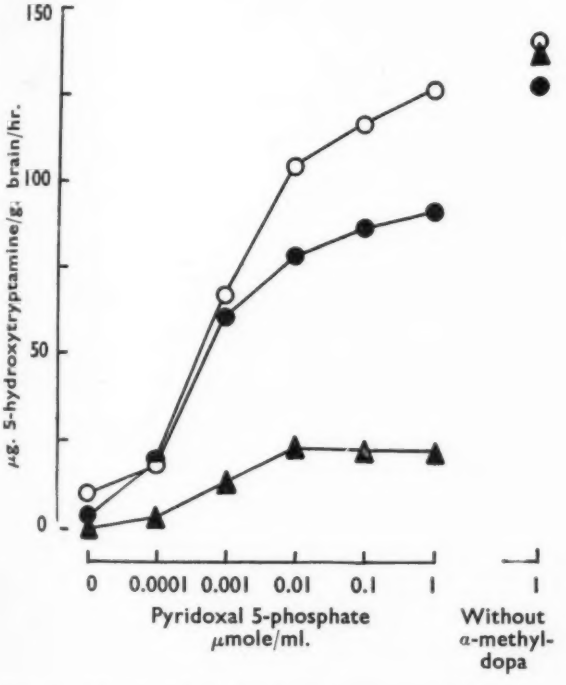

FIG. 2.—Mouse whole brain homogenate 5-hydroxytryptophan decarboxylase *in vitro*. Effect of varied 5-hydroxytryptophan and pyridoxal 5-phosphate concentrations in the presence of a fixed concentration of α-methyldopa 0.2 μmole/ml. incubation mixture. Iproniazid added 1 μmole/ml. Ordinates: μg. 5-hydroxytryptamine formed/g. brain/hr. Abscissae: pyridoxal 5-phosphate concentration in μmole/ml. 5-Hydroxytryptophan concentrations: O—O 10 μmole/ml. ●—● 1 μmole/ml. ▲—▲ 0.1 μmole/ml.

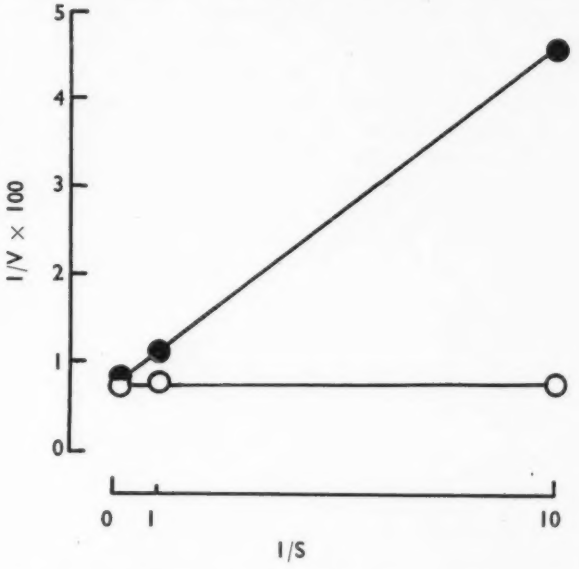

FIG. 3.—Mouse whole brain homogenate 5-hydroxytryptophan decarboxylase *in vitro*. Reciprocal analysis of activities in presence of pyridoxal 5-phosphate 1 μmole/ml. by method of Lineweaver and Burk (1934). See text for details. Ordinates: $1/V \times 100$, where V = reaction velocity in μg. 5-hydroxytryptamine formed/g. tissue/hr. Abscissae: 1/S, where S = substrate concentration in μmole/ml. ●—● with α-methyldopa 0.2 μmole/ml.; O—O without addition.

TABLE I

RECIPROCAL ANALYSIS OF RESULTS FROM FIG. 2

Intercepts and slopes for reciprocals of enzyme activity ($1/V \times 100$) against substrate concentration (1/S).

Inhibitor	Coenzyme Concentration in μmoles/ml.	Intercept ($1/V_{max}$)	Slope
−	0·0001 to 1	0·75	0
+	1	0·74	0·38
+	0·1	0·81	0·37
+	0·01	0·93	0·35
+	0·001	1·22	0·64
+	0·0001	3·87	2·93

the intercept is greater in the presence than in the absence of the inhibitor; this indicates non-competitive inhibition. At high coenzyme concentrations the intercept is identical whether inhibitor is present or not; this indicates competitive inhibition.

Action of Guinea-pig Kidney Decarboxylase on α-Methyldopa.—The preparation was incubated with various mixtures of 5-hydroxytryptophan and α-methyldopa at *p*H 8.0. Carbon dioxide evolution, measured by Warburg manometry, indicated that this enzyme preparation shows a much greater affinity for α-methyldopa than for 5-hydroxytryptophan at this *p*H, and that it decarboxylates α-methyldopa to a small extent. Fig. 4 shows the results obtained.

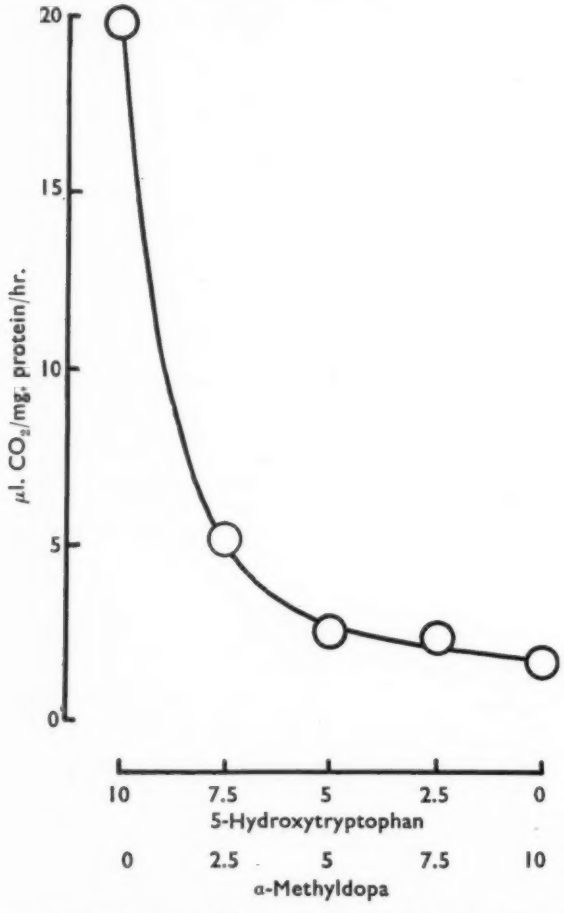

FIG. 4.—Guinea-pig kidney 5-hydroxytryptophan decarboxylase *in vitro*. Warburg manometry. Action on mixtures of 5-hydroxytryptophan and α-methyldopa. See text for details of preparation. Ordinates: μl. carbon dioxide evolved/mg. protein/hr. Abscissae: quantities of substrates added in μmole.

In Vivo *Experiments*

Mouse Brain and Intestinal Decarboxylase.—Groups of male white mice weighing 20 to 28 g. were given α-methyldopa 100 mg./kg. subcutaneously, and killed at intervals thereafter. Brain and intestinal homogenates were assayed for decarboxylase activity, without addition of pyridoxal 5-phosphate. The results, presented graphically in Fig. 5, show that activity was inhibited in both preparations, intestine being affected more than brain. Inhibition was maximal 0.5 to 1 hr. after treatment, and disappeared within 8 hr.

Mouse Brain and Intestinal 5-Hydroxytryptamine Concentration.—The results, given in Table II, show that α-methyldopa 100 mg./kg. sub-

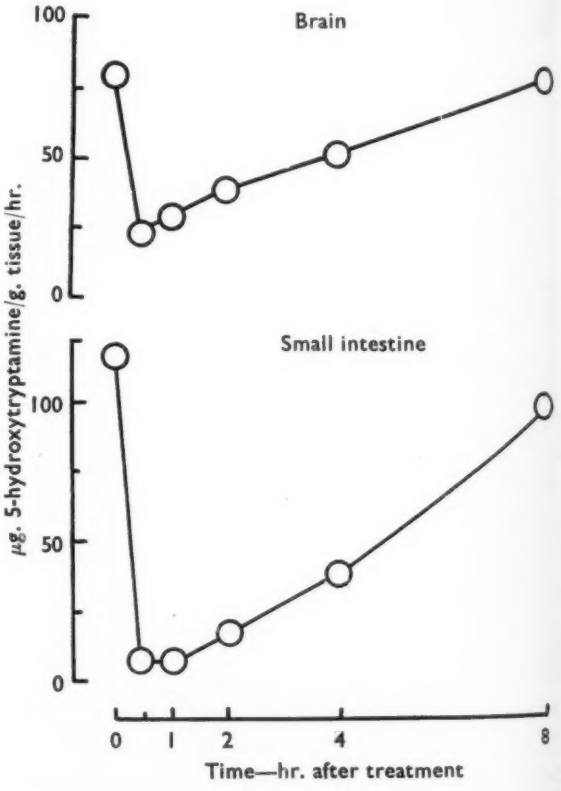

FIG. 5.—Mouse whole brain and small intestine homogenate 5-hydroxytryptophan decarboxylase activities after pretreatment with α-methyldopa 100 mg./kg. subcutaneously. No addition of pyridoxal 5-phosphate to incubation mixture. 5-Hydroxytryptophan and iproniazid concentrations each 1 μmole/ml. Each point obtained from pooled tissues of 2 mice. Ordinates: μg. 5-hydroxytryptamine formed/g. tissue/hr. Abscissae: time in hr. after treatment.

TABLE II

THE CONTENT OF 5-HYDROXYTRYPTAMINE IN MOUSE WHOLE BRAIN AND SMALL INTESTINE AFTER TREATMENT WITH α-METHYLDOPA

The figures represent μg. 5-hydroxytryptamine/g. tissue. Brain results were obtained on pairs of organs. Intestines were assayed singly.

Tissue	Saline 1 Dose at 1 hr.	α-Methyldopa 100 mg./kg. at 1 hr.	Saline 4-hourly for 24 hr.	α-Methyldopa 400 mg./kg. 4-hourly for 24 hr.
Brain	0·96 0·79 0·89	0·86 0·49 0·57	0·96 1·02	0·38 0·48
Intestine	2·51 1·77 2·64 3·17 3·29 3·61	3·79 3·55 2·29 3·01	3·05 5·07 3·33 3·22	2·82 2·61 1·81 2·00
	Mean =2·83 (±0·27)	Mean =3·16 (±0·33)	Mean =3·67 (±0·47)	Mean =2·31 (±0·24)
	p>0·4		p<0·05	

TABLE III

NORADRENALINE CONTENT OF MOUSE WHOLE BRAIN AFTER TREATMENT WITH α-METHYLDOPA

The figures represent μg. noradrenaline/g. brain.

Saline 1 Dose at 1 hr.	α-Methyldopa 100 mg./kg. at 1 hr.	Saline 4-hourly for 24 hr.	α-Methyldopa 400 mg./kg. 4-hourly for 24 hr.
0·90 0·80	0·95 0·80	0·82 1·04	0·92 1·00

TABLE IV

5-HYDROXYTRYPTAMINE CONTENT OF GUINEA-PIG WHOLE BRAIN AFTER TREATMENT WITH α-METHYLDOPA

The figures represent μg. 5-hydroxytryptamine/g. brain.

Saline 4-hourly for 24 hr.	α-Methyldopa 400 mg./kg. 4-hourly for 24 hr.
0·30 0·28	0·03 0·02

cutaneously reduced the brain 5-hydroxytryptamine content, the reduction (about 30%) being maximal 1 hr. after treatment. A much bigger reduction (60%) was found with a larger dose (400 mg./kg.) given at 4-hourly intervals for 24 hr. Intestinal 5-hydroxytryptamine concentrations were not affected by the single doses, but were reduced by about 40% by the repeated doses of α-methyldopa.

Mouse Brain Noradrenaline Concentration.—α-Methyldopa in a single dose of 100 mg./kg. or in multiple dosage of 400 mg./kg. 4-hourly for 24 hr. had no effect on noradrenaline concentrations in the whole brain. Table III shows the results found.

Guinea-pig Brain 5-Hydroxytryptamine Concentration.—α-Methyldopa 400 mg./kg. 4-hourly for 24 hr. produced more than 90% reduction in whole brain 5-hydroxytryptamine content. This reduction was much greater than was found in mice. The results are given in Table IV.

Pharmacological Effects

Mouse Activity.—Groups of mice treated with α-methyldopa 100 mg./kg. subcutaneously were found to be much less active than saline-treated controls. This is shown in Fig. 6. The activity was minimal 1 hr. after treatment, at which time the animals were considerably sedated. Eight hr. after treatment the animals appeared normal and the activity counts had returned to the control level.

In further experiments, the results of which are shown in Fig. 7, groups of mice were treated with α-methyldopa (100 mg./kg.) and/or 5-hydroxytryptophan (100 mg./kg.) or dopa (90 mg./kg.), both of which are substrates for decarboxylase and might be expected to reduce the inhibition produced by α-methyldopa. Other groups received α-methyldopa (100 mg./kg.) and/or iproniazid (50 mg./kg.) or harmaline (20 mg./kg.), both of which are amine oxidase inhibitors and might be expected to prevent destruction of 5-hydroxytryptamine in the brain. All treatment was given subcutaneously, iproniazid 4 hr. before, and all other substances 1 hr. before testing. 5-Hydroxytryptophan reduced activity slightly, though not significantly, but diminished the effect of α-methyldopa ($p<0.05$). Dopa produced a big reduction ($p<0.01$) and exacerbated the effect of α-methyldopa ($p<0.01$). Iproniazid had no effect alone but reduced the effect of α-methyldopa ($p<0.05$). Harmaline produced a big reduction in activity ($p<0.001$), but did not influence the effect of α-methyldopa.

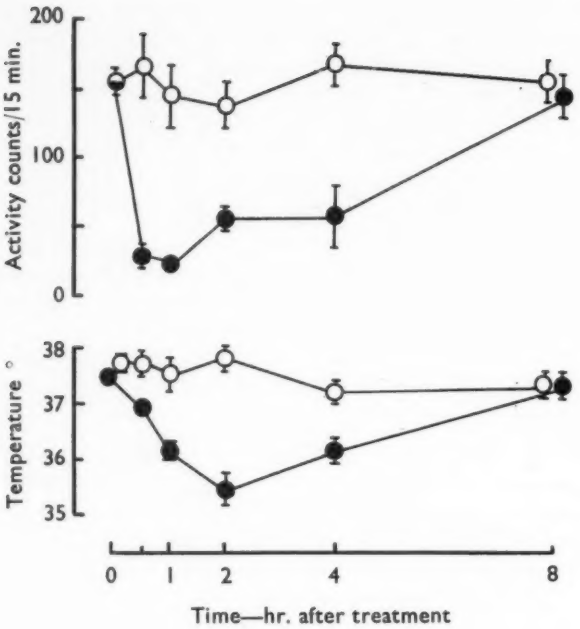

FIG. 6.—Activity counts and rectal temperatures of mice after treatment with α-methyldopa or saline in equivalent volume. Vertical lines show standard errors of the mean. Ordinates—upper graph: mean activity counts of 3 groups of mice/15 min.; lower graph: mean rectal temperature of 10 mice in °. Abscissae: time in hr. after treatment. O—O saline treatment. ●—● α-methyldopa 100 mg./kg.

Mouse Rectal Temperature.—The temperature of mice treated with α-methyldopa 100 mg./kg. subcutaneously was reduced by comparison with saline-treated controls. This is shown in Fig. 6. The temperature fall continued after the maximal reduction in activity, and was greatest 2 hr. after treatment. In further experiments, groups of mice were treated with combinations of α-methyldopa and/or 5-hydroxytryptophan, dopa, iproniazid, or harmaline. Doses and timing were exactly as in the experiments described for activity, and the difference in temperatures from saline-treated animals are shown in Fig. 7. 5-Hydroxytryptophan reduced temperature ($p<0.001$) but did not alter the effect of α-methyldopa significantly. Dopa had no significant action alone, but it exacerbated the effect of α-methyldopa ($p<0.01$). Iproniazid had no action at all. Harmaline reduced temperature ($p<0.001$) and exacerbated the effect of α-methyldopa ($p<0.01$).

Guinea-pig Rectal Temperature.—α-Methyldopa 400 mg./kg. 4-hourly for 24 hr. had a slight effect only on rectal temperature of guinea-pigs. Figures obtained were:

Before treatment	..	37.6°, 37.4°
After treatment	..	36.4°, 36.9°

Other Drugs on Mouse Rectal Temperature.—Hexobarbitone sodium 80 mg./kg. intraperitoneally produced a fall in temperature which was maximal 0.5 to 1 hr. after treatment. The mean decrease at 1 hr. was 2.6° ($p<0.05$). Reserpine 5 mg./kg. subcutaneously produced at 24 hr. a fall in temperature which was usually more than 7.5°, that is too low to be measurable with the thermistor circuit as calibrated.

Intestinal Passage of a Meal.—Test meals of carmine were given to a group of mice 1 hr. after

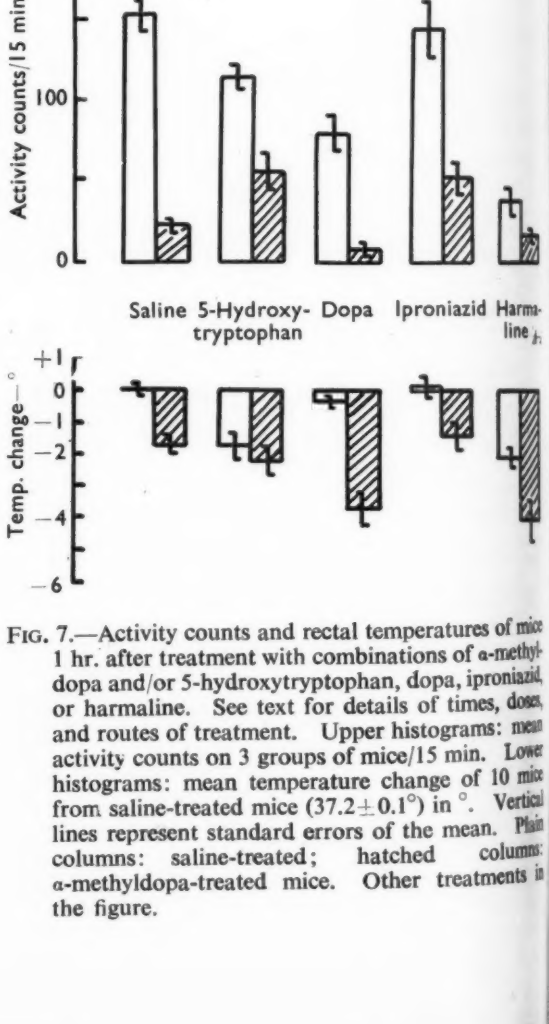

FIG. 7.—Activity counts and rectal temperatures of mice 1 hr. after treatment with combinations of α-methyldopa and/or 5-hydroxytryptophan, dopa, iproniazid, or harmaline. See text for details of times, doses, and routes of treatment. Upper histograms: mean activity counts on 3 groups of mice/15 min. Lower histograms: mean temperature change of 10 mice from saline-treated mice (37.2±0.1°) in °. Vertical lines represent standard errors of the mean. Plain columns: saline-treated; hatched columns: α-methyldopa-treated mice. Other treatments in the figure.

TABLE V

PASSAGE OF CARMINE MEAL IN MICE AFTER VARIOUS TREATMENTS

Details of dosage and routes of treatment in text. Figures are means (±standard error). Figures in italics —$p<0.02$.

Treatment	No. of Animals	% of Carmine Remaining in the Stomach	% of Small Intestine Traversed by the Meal
Saline ..	12	21 (±6)	73 (±3)
α-Methyldopa	12	*55* (±7)	*55* (±3)
Atropine ..	6	50 (±10)	*55* (±8)
Controls for reserpine ..	12	32 (±5)	69 (±5)
Reserpine ..	12	*19* (±2)	*46* (±4)

treatment with α-methyldopa 100 mg./kg. subcutaneously. The results, given in Table V, show that α-methyldopa delayed gastric emptying, for the amount of carmine remaining in the stomach was greater than in the control animals. The percentage of the small intestine traversed by the meal was less after α-methyldopa. Atropine 20 mg./kg. subcutaneously 1 hr. previously had exactly the same effect.

Two groups of mice were given reserpine (in ascorbate solution) 5 mg./kg. or ascorbate solution subcutaneously and starved for 24 hr. Gastric emptying was more complete in the reserpine-treated animals, but in spite of this the percentage of the small intestine traversed by the meal was less.

Hexobarbitone Sleeping Time.—Groups of 10 mice were given α-methyldopa 100 mg./kg. subcutaneously or saline, followed 30 min. later by hexobarbitone sodium 80 mg./kg. intraperitoneally. The mean sleeping times, calculated as the interval between injection and the time of restoration of the righting reflex (± standard error), were:

Hexobarbitone .. .. 20 (±2) min.
α-Methyldopa + hexobarbitone 43 (±6) min.

These results were significantly different ($p<0.01$).

Eye Signs.—Narrowing of the palpebral fissure occurred in all mice treated with α-methyldopa, whether in a single dose or multiple doses, as previously described. Miosis was present in those animals which were treated repeatedly. Both these signs were present to a very marked degree in 2 guinea-pigs given 4 hourly doses of α-methyldopa 400 mg./kg.

Similarities Between Appearances of Animals Treated with Reserpine and α-Methyldopa.—In many respects the appearance of α-methyldopa-treated and reserpine-treated animals was very similar. Both were sedated, inactive, and hypothermic, and the eye signs were the same. One difference, however, was noted. Twenty-four hours after reserpine, mice and guinea-pigs, though sedated, react violently to any handling. This was not so after repeated doses of α-methyldopa. The animals remained very quiet and limp, and were apparently quite oblivious of their surroundings.

DISCUSSION

The Action of α-Methyldopa on 5-Hydroxytryptophan Decarboxylase. — The experiments *in vitro* showed that the effect of α-methyldopa depends on a relationship between the concentrations of inhibitor, substrate, and coenzyme. The most significant finding is that increased concentrations of either substrate or coenzyme or both reverse the inhibition. At high coenzyme concentrations the relationship between substrate and inhibitor is one which indicates a competitive type of inhibition, a concept which is supported by the evidence that α-methyldopa is itself decarboxylated, albeit at a slow rate. At low coenzyme concentrations, however, a different mechanism probably exists. Reciprocal analysis of the results showed that inhibition was partly non-competitive, and the fact that raising the coenzyme concentration was capable of reversing the inhibition suggests that α-methyldopa can inactivate coenzyme. Whether this inactivation is caused by a chemical reaction between the two is not clear. Schott and Clark (1952) and Holtz and Westermann (1957) showed that a number of decarboxylase inhibitors, derivatives of phenylalanine and phenylethylamine, react with pyridoxal, and that the reaction is dependent on the presence of a hydroxyl group in the *meta*-position. They showed that the reaction takes place in two stages, to form first a Schiff base and then a tetrahydroisoquinoline derivative by ring closure. However, Sourkes (1954) demonstrated only a very slow combination between pyridoxal and α-methyldopa, though this substance does contain a *meta*-hydroxyl group. The mechanism of the coenzyme reversal of α-methyldopa inhibition is, therefore, incompletely explained.

One of the implications of the *in vitro* findings is that the measurement of inhibition *in vivo* can never represent a true picture of what occurs in

the tissues themselves. Knowledge of local concentrations of all the variables is necessary before any assumptions can be made. In any case, all known methods of enzyme activity measurement involve the addition to the incubation mixture of relatively large amounts of substrate, so that the substrate/inhibitor ratio is already disturbed. From this viewpoint, the present measurements of decarboxylase inhibition *in vivo* provide only one piece of information: that α-methyldopa reaches the brain. The only absolute indication of inhibition *in vivo* is that brain and intestinal 5-hydroxytryptamine concentrations are reduced.

Tissue Amine Concentrations.—The finding that α-methyldopa treatment led to a reduction in brain 5-hydroxytryptamine but not in brain noradrenaline is of considerable interest. This might be due to a preferential action on 5-hydroxytryptophan as opposed to dopa decarboxylase. Westermann *et al.* (1958) demonstrated such a preference in their experiments, and the possibility that local concentrations of the two substrates in the brain may differ might contribute to this difference. On the other hand, it must be remembered that 5-hydroxytryptamine and noradrenaline do not lie in equivalent biochemical relation to decarboxylase. Whereas 5-hydroxytryptamine is itself the product formed from 5-hydroxytryptophan, dopamine is the product formed from dopa, and noradrenaline is only formed by β-hydroxylation of dopamine. The fact that there are considerable quantities of dopamine present in the brains of different species (Carlsson, Lindqvist, Magnusson, and Waldeck, 1958; Bertler and Rosengren, 1959) suggests that this β-hydroxylation is a limiting reaction by comparison with the decarboxylation which precedes it. It is perhaps not surprising, therefore, that noradrenaline concentrations were not affected in the present experiments. So far, no estimations of dopamine have been performed here, but these are planned for the near future. The effect of α-methyldopa on them should be of great interest.

Pharmacological Effects of α-Methyldopa.—It is evident that α-methyldopa produced many of the effects of reserpine, though presumably in a completely different manner. The implications of this finding are considerable. Reserpine is known to deplete the brain of 5-hydroxytryptamine (Hess, Shore, and Brodie, 1956), noradrenaline (Vogt, 1957; Shore, Mead, Kuntzman, Spector, and Brodie, 1957), and dopamine (Carlsson *et al.*, 1958), and it has been suggested at various times that these depletions are responsible for the sedative and ocular effects that reserpine produces. Hess *et al.* (1956) suggested that these effects are due to the presence of free 5-hydroxytryptamine acting on the brain. The present experiments make this very unlikely, as it is inconceivable that inhibition of decarboxylase should cause the presence of free 5-hydroxytryptamine. The experiments also suggest that the depletion of noradrenaline is of less importance than that of 5-hydroxytryptamine, for α-methyldopa had no effect on noradrenaline concentrations. The possibility remains that depletion of dopamine has occurred also after α-methyldopa. Whether this has contributed to its pharmacological effects remains to be seen.

The experiments in which 5-hydroxytryptophan, dopa, iproniazid, or harmaline were given in addition to α-methyldopa illustrate some points of interest. 5-Hydroxytryptophan alone reduced activity to a slight extent and it is possible that a high concentration of this substrate in the brain inhibits decarboxylase by coenzyme removal. This has been found *in vitro* with dopa (Schott and Clark, 1952). On the other hand, 5-hydroxytryptophan diminished the reduction in activity produced by α-methyldopa, presumably reversing its inhibitory action on the enzyme. By contrast, dopa had the same effect as, and potentiated the action of, α-methyldopa. This suggests that dopa inhibits 5-hydroxytryptophan decarboxylase *in vivo* as it does *in vitro* (Yuwiler, Geller, and Eiduson, 1959), and that the effects of dopa and α-methyldopa are, in this respect, additive. Iproniazid diminished the effect of α-methyldopa, as would be expected if activity is related to the concentration of 5-hydroxytryptamine in the brain. The effects of harmaline were unexpected and suggest that this substance has some other action beside its amine oxidase inhibitory one. What this action is remains obscure.

The temperature changes in all the treated mice followed very approximately the observed changes in activity. This suggests that the two are related. That falls in temperature were consequent upon inactivity, and not vice versa, is suggested by two findings: first, that temperatures of α-methyldopa treated mice continued to fall from 1 to 2 hr. after treatment, at a time when activity counts were already returning to normal; and secondly, that guinea-pigs treated with α-methyldopa were sedated and showed marked eye signs, though their temperatures fell only very slightly. This difference may be a reflection of the different surface area/heat capacity ratios in the two

species, a factor which would make mice particularly prone to changes in temperature. Lessin and Parkes (1957) showed that keeping mice at an ambient temperature of 32° prevents the sedative action of reserpine, and concluded that the sedation was a result of the hypothermia. The present findings are not in keeping with such an idea.

The experiments on the passage of a test meal in mice showed that α-methyldopa treatment produced an apparent slowing, thus imitating the effect of reserpine. There was, however, one striking difference between the effects of the two substances. Whereas reserpine produced a slight acceleration of gastric emptying, α-methyldopa delayed it. The effects of α-methyldopa were exactly mimicked by those of atropine, which suggests that they might have been mediated via the vagus and possibly resulted from a decrease in parasympathetic tone.

The Use of Decarboxylase Inhibitors.—The use of α-methyldopa, and probably other substances with similar actions, provides a method of depleting tissue of 5-hydroxytryptamine without the use of releasing agents such as the Rauwolfia alkaloids. Also, its greater effect on 5-hydroxytryptamine as opposed to noradrenaline formation provides a way of distinguishing some of the functions of these two amines in the body. Lastly, it provides a possible mode of treatment for human subjects suffering from the effects of excessive 5-hydroxytryptamine production by carcinoid tumours.

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EFFECT OF DENERVATION AND OF COCAINE ON THE ACTION OF SYMPATHOMIMETIC AMINES

BY

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The secretory effect of sympathomimetic amines on the submaxillary gland of cats was increased after section of the chorda tympani (preganglionic, parasympathetic supply). After sympathetic denervation of the gland the secretory response to tyramine and phenylethylamine was absent, the response to dopamine and ephedrine decreased and the response to adrenaline and noradrenaline increased. Large doses of cocaine, given locally into the gland, produced changes similar to those observed after sympathetic denervation. The sensitization towards adrenaline and noradrenaline was obtained with smaller doses. Tyramine did not cause a release of catechols from the suprarenal glands of the cat.

It is known that sympathetic denervation has different effects on the response of the nictitating membrane to different sympathomimetic amines (Fleckenstein and Burn, 1953). Some amines are no longer effective shortly after denervation and these are considered not to have a direct action upon the effector organ. The response to other amines is increased, and these are supposed to exert a direct action on the effector organ. Thirdly, there is an intermediate group of substances which exert some effect even after denervation; these are believed to have both a direct and indirect mode of action.

In a number of tissues cocaine affects the response to sympathomimetic amines in the same way as sympathetic denervation: the effect of an indirectly acting substance, like tyramine, is decreased, while the response to substances which act directly, like adrenaline, is increased (Fröhlich and Loewi, 1910; Tainter and Chang, 1927; Burn and Tainter, 1931; Burn, 1932; Emmelin and Muren, 1951; Fleckenstein and Bass, 1953; Fleckenstein and Stöckle, 1955).

The salivary glands offer a special advantage for a comparison of the effects of cocaine and denervation; a high dose of cocaine can be given locally, via the secretory duct, to affect only one of the two submaxillary glands.

METHODS

Cat submaxillary glands were denervated sympathetically by excision of the superior cervical ganglion under ether anaesthesia. Acute experiments were carried out under chloralose anaesthesia.

The drugs were given intravenously into the femoral vein or via a cannula in the lingual artery (in 0.1 ml. saline during 10 sec.) after ligating all arterial branches except that to the submaxillary gland. When substances were given by the submaxillary duct, the injections were done as described by Emmelin, Muren, and Strömblad (1954).

The drops of saliva falling from the cannula inserted in the submaxillary duct were recorded by a signal on a smoked drum. The cannulae used delivered 1 ml. of distilled water in 40 drops.

The suprarenal glands were perfused according to Emmelin and MacIntosh (to be published).

RESULTS

Secretory Responses Due to Drug Action.—The effects of DL-phenylalanine, DL-tyrosine, dopa, β-phenylethylamine, tyramine, dopamine (3-hydroxytyramine) and l-ephedrine were studied. The three amino acids, tested by intravenous injection, phenylalanine (20 mg./kg.), tyrosine (20 mg./kg.), and dopa (2 mg./kg.), did not have any secretory activity.

With phenylethylamine, tyramine, dopamine, and ephedrine a secretion was obtained both by intravenous and close-arterial injection. The threshold doses, in normally innervated submaxillary glands, for intravenous injections were: for phenylethylamine about 5 mg./kg., for tyramine and dopamine 50 to 200 μg./kg. and for ephedrine about 2 mg./kg.

The responses refer to the first dose given; it was found that all these compounds exhibited

tachyphylaxis and cross-tachyphylaxis. In Fig. 1 an experiment is shown in which cross-tachyphylaxis between phenylethylamine and tyramine occurred. This figure is taken from an experiment on an animal in which both chordae tympani had been cut three weeks earlier; this led to the sensitization of the glands to these substances which will be discussed below. Phenylethylamine (1 mg.) was given close-arterially to the left gland and 14 drops were secreted; the same dose given to the right gland led to a secretion of 9 drops. After a second dose of phenylethylamine to the left gland, the response was 12 drops, that is, a slightly reduced amount. An intravenous infusion of tyramine was then started, with 100 mg. given during 1 hr. When a second dose of phenylethylamine was then applied to the right gland no secretion occurred. Acetylcholine, tested before and after the infusion, gave identical responses from the right gland. Thus the tyramine had inhibited the response to phenylethylamine on the right side; without the infusion the effect would only have been diminished by as little as on the left side.

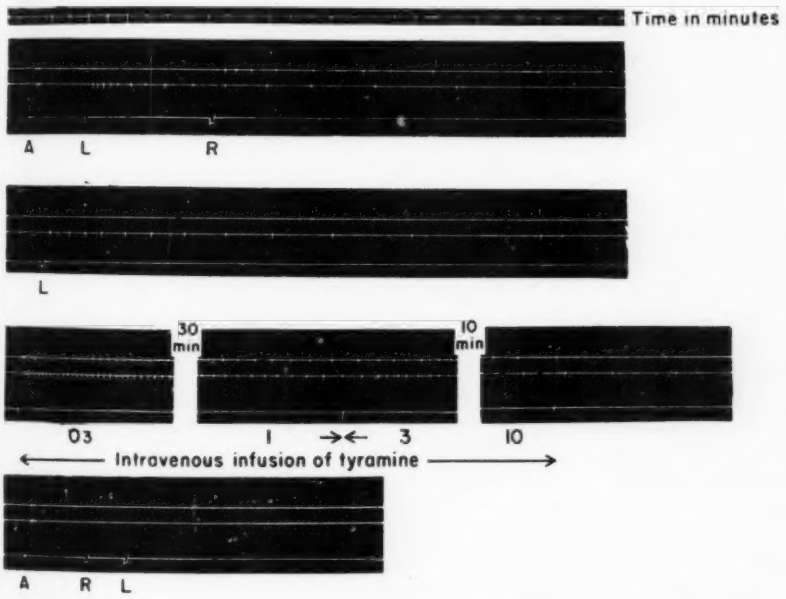

FIG. 1.—Cross-tachyphylaxis for phenylethylamine and tyramine in cat submaxillary gland. Cat 2.9 kg. Right and left chorda tympani cut 3 weeks earlier. Top, time tracing in min. In each of the other tracings the three records are from above: secretion, in drops, from right gland; secretion, in drops, from left gland; signal. A, Injection of 10 μg./kg. of acetylcholine intravenously. L and R: injection into the left (L) and right (R) lingual artery of 1 mg. of phenylethylamine during 10 sec. in a volume of 0.1 c.c. The infusion rate of tyramine in mg./min. is given under the record. A total of 100 mg. tyramine was infused during one hour.

Similar results were obtained when dopamine or l-ephedrine were tested in single doses with tyramine infusions, and also with tyramine as the test substance and an infusion of 100 mg. ephedrine in 1 hr. A single dose of ephedrine (3 mg./kg.) did not abolish the response to tyramine.

Section of Chorda Tympani.—Three to five weeks after section of the parasympathetic (preganglionic) supply to the submaxillary gland, supersensitivity was always present towards phenylethylamine, dopamine, tyramine, and ephedrine. In a cat, operated on one side three weeks before the experiment, the secretory response to an intravenous injection of 5 mg./kg. of phenylethylamine was 27 drops on the operated side and a just perceptible secretion, amounting to less than a drop, on the normally innervated side. Dopamine (100 μg./kg.), tested in another animal, gave 25 drops on the operated, and a trace of secretion on the normally innervated side. Similar observations have already been reported for ephedrine (Emmelin and Muren, 1951) and for tyramine (Strömblad, 1956).

Sympathetic Denervation.—Three weeks after ganglionectomy the secretory responses to both tyramine and phenylethylamine were abolished even when these substances were given in large doses (up to 5 mg./kg. of tyramine and 20 mg./kg. of phenylethylamine). An acute excision of the superior cervical ganglion did not affect the secretory response of the gland.

A secretion from the gland in response to dopamine and ephedrine was still obtained three weeks after sympathetic denervation. With these substances, the threshold on the denervated side was lower than on the innervated side, but the secretory response to larger doses was greater for the gland with its sympathetic nerve supply intact.

Effects of Cocaine.—Advantage was taken of the increased responsiveness of the submaxillary gland after section of the chorda tympani. In these experiments, cocaine was

given either intravenously or by the glandular duct. It is known that 2 mg./kg. of cocaine, given intravenously, causes a supersensitivity to adrenaline and noradrenaline (Emmelin and Muren, 1951). This was confirmed in the present experiments. On the other hand, it was found that the response to tyramine decreased. In two cats previously given cocaine (2 mg./kg.) intravenously, 100 μg./kg. of tyramine did not cause any secretion; this is a dose to which all other parasympathetically denervated glands had responded. After this dose of cocaine there was, however, no complete loss of responsiveness to tyramine, since 500 μg./kg. of tyramine still caused a flow of saliva. No detailed quantitative analysis of the effect of cocaine given intravenously was attempted in view of the tachyphylaxis already referred to.

Cocaine, 0.1 mg. administered by the submaxillary duct, regularly caused a supersensitivity to adrenaline and noradrenaline. A further increase in sensitivity was usually noted when 1 mg. of cocaine was given by the same route; when this dose was followed, in 2 cats, by an intravenous injection of 2 mg./kg. of cocaine, no further increase in sensitivity was seen. In 3 cats, 3 successive injections of cocaine, 0.1 mg., 1 mg., and 10 mg., were made in intervals of 1 hr. In one of these experiments, the secretory responses were measured after 5 μg./kg. of noradrenaline intravenously, and after sympathetic stimulation for 1 min. they were as follows:

	After noradrenaline	After sympathetic stimulation
Before cocaine	1 drop	16 drops
After 0.1 mg. cocaine ...	15 drops	14 „
After further 1 mg. cocaine	23 „	15 „
After further 10 mg. cocaine	19 „	0 „

Thus the sensitization of the response to noradrenaline was fully developed with doses of cocaine much lower than those necessary to block completely the effect of sympathetic stimulation.

Tyramine was similarly tested. Secretion was seen as long as stimulation of the sympathetic nerve was effective. When no secretion occurred on stimulation of the sympathetic, even large doses of tyramine were ineffective; it was confirmed that small doses of acetylcholine were still effective.

In some of the experiments, the large doses of cocaine necessary to block the effect of sympathetic stimulation also abolished the response to acetylcholine; such experiments were discarded. To avoid such an unspecific effect of cocaine, a procedure was eventually adopted in which the big dose of cocaine was replaced by two smaller doses given with a time interval. The following observations were made in one of these experiments: before any cocaine was given the secretion after 20 μg./kg. of noradrenaline intravenously was 1 drop on each side. After 5 mg. of cocaine given by the right submaxillary duct, the response of the right gland to the same dose of noradrenaline was 20 drops, the response from the left gland was 2 drops. One hour later 5 mg. of cocaine was given, via the duct, on the right side. The response to the intravenous test dose of noradrenaline was now 17 drops from the right gland and 19 drops from the left gland. Obviously, by now cocaine had reached the left gland and had rendered it supersensitive to noradrenaline. Sympathetic stimulation on the right side was now tested and found to be ineffective, but on sympathetic stimulation of the left side, secretion of saliva occurred. Tyramine (1 mg./kg. intravenously) was then tested: 6 drops of saliva were counted on the left side; no secretion occurred on the right side. This experiment reinforces the conclusion drawn from the experiments already described: with cocaine it is possible to achieve maximal sensitization towards adrenaline and/or noradrenaline without blocking the response to sympathetic stimulation. The experiment also shows that a block of the sympathetic nerve by cocaine abolishes the effect of tyramine.

The responses to both dopamine and ephedrine were diminished, but not abolished, when the response to sympathetic stimulation was blocked by cocaine. The effects of section of the chorda tympani, of sympathetic denervation, and of small and big doses of cocaine on the secretory response to various amines are summarized in Table I.

Perfusion of the Suprarenal Glands.—It has been suggested by Burn and Rand (1958a) that the action of tyramine is mediated by a release of catecholamines. It was therefore of interest to study the effect of tyramine on the perfused suprarenal gland, in order to find out if in this location evidence could be obtained for a releasing action of tyramine.

Three experiments were performed in which the suprarenal glands of cats were perfused with oxygenated Tyrode solution. Each perfusion lasted 5 min.; the volume perfused was between 4 and 6 ml. For two periods in each animal, either 1 μg./ml. or 10 μg./ml. or 100 μg./ml. of

TABLE I

THE EFFECT OF VARIOUS PROCEDURES ON THE SECRETORY RESPONSE FROM THE SUBMAXILLARY GLAND OF CATS

+ = Increased secretory response, − = decreased secretory response, +− = increased response to small doses and decreased response to bigger doses, 0 = no secretory effect even with very big doses.

Drug	Threshold Dose on Normally Innervated Glands	Secretory Response After			
		Para-sympathetic Denervation	Sympathetic Denervation	Cocaine 2 mg. Intravenously	Cocaine 10 mg. into the Sub-maxillary Duct
β-Phenylethylamine ..	2–5 mg./kg.	+	0	−	0
Tyramine	50–200 μg./kg.	+	0	−	0
Dopamine	50–200 ,,	+	+−	+−	+−
l-Ephedrine	2–5 mg./kg.	+	+−	+−	+−
Noradrenaline	5–50 μg./kg.	+	+	+	+
Adrenaline	5–50 ,,	+	+	+	+

tyramine were added. The perfusates were tested against adrenaline on the cat submaxillary gland with the chorda tympani cut and the superior cervical ganglion excised 3 weeks before; in this way a contributory effect on the gland of the tyramine present in the perfusate could be avoided.

In one of these experiments the activity found in the perfusates, expressed in μg./min. of adrenaline, was as follows:

Perfusion Fluid
Tyrode 1.2 (1st period) 0.9 (5th period)
Tyrode containing 1 μg./ml. of tyramine 0.9 (2nd period) 0.8 (6th period)
Tyrode containing 10 μg./ml. of tyramine 1 (3rd period) 0.9 (7th period)
Tyrode containing 100 μg./ml. of tyramine 1 (4th period) 0.6 (8th period)

The results obtained with the two other animals were very similar to those just given. The activity of the perfusates was not affected by atropine (1 mg.), but it was blocked by dihydroergotamine (1 mg.).

DISCUSSION

Excision of the superior cervical ganglion affects the secretory response of the submaxillary gland as well as the response of the nictitating membrane to sympathomimetic amines. Tyramine and phenylethylamine completely lost the power of causing secretion, while, as is well known, the secretory responses to adrenaline and noradrenaline were augmented. Dopamine and ephedrine represented an intermediate group: doses near the threshold were more effective after sympathetic denervation, while bigger doses were less effective than on normally innervated glands; this could be explained by assuming that the denervated gland had become sensitized to the direct effect of these two compounds, but that the indirect action had been abolished.

The observations on cocaine are of particular interest. In a high dose cocaine not only blocked the effects of sympathetic stimulation, but it also had other effects identical with those seen after excision of the superior cervical ganglion, that is, a sensitization of the gland to the direct effects of the amines and an abolition of their indirect actions. Sensitization was also fully developed after small doses of cocaine, but these did not extinguish the effects of substances believed to act indirectly. These observations are in accordance with those of Fleckenstein and Stöckle (1955) on the nictitating membrane; these authors found it possible to sensitize the membrane to substances like adrenaline or noradrenaline with smaller doses of cocaine than are necessary to diminish the response to indirectly acting substances, for example, tyramine. These two effects are probably brought about by different mechanisms because different doses of cocaine are required to cause a super-sensitivity to noradrenaline on the one hand, and to abolish the response to tyramine on the other.

These mechanisms are unknown, but the experiments reported demonstrate that a functionally intact sympathetic supply is necessary for the indirect effect of tyramine. Experiments with reserpine (Burn and Rand, 1958a) suggest that a local store is required for the indirect action. Consequently, it was suggested that tyramine and similar amines act by release of catechol amines.

It is conceivable that cocaine acts on these stores, which presumably are located in or near the nerve terminals. The experiments reported suggest that the sensitization of the effector organ to adrenaline and similar compounds is not brought about by the same mechanism.

In the experiments on the perfused suprarenal glands, tyramine did not cause a release of catechol amines. Thus, either the indirect action of tyramine and similar amines is not to release catechol amines, as discussed above, or the mechanism for release in the medulla of the suprarenal gland differs from that in the submaxillary gland and the nictitating membrane.

It seems possible that the chromaffin tissue present in the adrenal medulla, or in the skin of the rabbit ear (Burn and Rand, 1958b), or in the human skin (Adams-Ray and Nordenstam, 1956) represents one type of store that can be released by nicotine; this has already been discussed elsewhere (Strömblad, 1959). The adrenergic neurones, on which tyramine and similar substances might act as releasers, may represent a second type of store of catechol amines. Some support for this hypothesis might be found in the experiments by Ginzel and Kottegoda (1953), who observed that the constrictor action of nicotine in the rabbit ear was still present three weeks after sympathectomy. On the other hand, the present experiments have shown that the effect of tyramine is abolished by factors which interfere with the sympathetic nervous supply.

The finding of a cross-tachyphylaxis for all the sympathomimetic amines which act indirectly suggests a similar mechanism of action for all these compounds.

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EFFECTS OF ERGOMETRINE (ERGONOVINE) ON THE ISOLATED ATHEROSCLEROTIC HEART OF THE CHOLESTEROL-FED RABBIT

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Ergometrine usually depresses the S-T segment as in coronary insufficiency, when injected intravenously in rabbits with experimental coronary atherosclerosis and in patients with effort angina, but not in normal animals and man. To explain this difference, we carried out Langendorff perfusion studies in 32 normal and 29 atherosclerotic isolated rabbit hearts. Preliminary tests with ergometrine were done to ensure that advanced coronary atherosclerosis had developed in the rabbits fed a cholesterol diet; pathological examination of the heart after perfusion confirmed the result of the final test with ergometrine. Before drugs were perfused, the basal rate of coronary flow was greater, the heart rate was slower and the contractile amplitude was smaller in the atherosclerotic than in the normal hearts; nitroglycerin markedly increased flow in both normal and atherosclerotic groups. Ergometrine consistently caused a reduction in contractile amplitude with negligible changes in heart rate in both normal and atherosclerotic hearts. On coronary flow, however, the effects of ergometrine differed significantly in these groups; in doses of between 0.2 and 0.4 mg., the average decrease in flow was 8% in normal and 22% in atherosclerotic hearts. The effect was more variable in normal hearts and an increase in flow sometimes occurred. The difference in the response of normal and atherosclerotic hearts was particularly striking when ergometrine was given during recovery from a reduction of coronary flow which had been induced by vasopressin. Ergometrine then uniformly increased flow in the normal, but usually had the opposite effect in the atherosclerotic heart. In normal and atherosclerotic hearts, cardiac effects of vasopressin were similar. Tachyphylaxis to vasopressin, but not to ergometrine, was observed.

Ergometrine injected intravenously produces transient S-T segment depression characteristic of coronary insufficiency in patients with coronary artery disease (Stein, 1949; Stein and Weinstein, 1950, 1953; Rinzler, Stein, Bakst, Weinstein, Gittler, and Travell, 1954), and in rabbits with experimental coronary atherosclerosis (Rinzler, Travell and Karp, 1955; Rinzler, Travell, Karp, and Charleson, 1956). Such electrocardiographic changes after ergometrine are not seen in man or animal with a normal coronary circulation.

Although effects of ergometrine on heart rate and amplitude had been investigated in the normal frog (Davis, Adair, Chen, and Swanson, 1935), rabbit (Brown and Dale, 1935) and cat (Rothlin, 1935), we found no reports concerning the actions of this drug directly on the coronary circulation. We therefore investigated the mechanism of the electrocardiographic effects of ergometrine by perfusion of the isolated heart in normal rabbits and in those rendered atherosclerotic by cholesterol feeding.

It was found that the atherosclerotic isolated heart can be satisfactorily perfused and serves like the normal heart for studying drug effects on coronary flow, heart rate, and amplitude of contraction. Furthermore, satisfactory pathological detail of the coronary arteries is obtained on section of the heart after perfusion. Preliminary reports (Karp, Penna, Rinzler, and Travell, 1956; Karp, Travell, and Rinzler, 1956; Travell, Karp, and Rinzler, 1957) have been made of what we believe are the first perfusion studies of the isolated atherosclerotic heart. The present

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report deals with the methods, the state of the isolated atherosclerotic heart, and the effects of ergometrine on this preparation.

METHODS

Animal Care.—Male Dutch rabbits (Loryctolageus Cuniculus Domesticus) were housed in single cages in an air-conditioned room. They were placed first on a stock diet for 10 days, and if conjunctivitis, coryza, or diarrhoea developed, they were given sulphamethylthiazole (Sulmet-Lederle) intraperitoneally every other day for 3 doses of 200 mg. each. Two groups of rabbits were then started at the same time either on the stock diet or on a similar diet with added cholesterol. The diets were fed ad libitum.

Stock and cholesterol diets were commercially prepared (Vi-D-Co). The stock diet comprised: rolled oats 7.5 g.; ground corn 27.6 g.; dried skim milk 7.5 g.; grey shorts 7.5 g.; 17% dehydrated alfalfa 37.2 g.; bone meal 2.0 g.; calcium carbonate 1.5 g.; mineral salt 1.0 g. Either 2% cholesterol with 6% corn oil, or 2% cholesterol alone, was added to the stock diet. When the rabbits nibbled pellets prepared with corn oil, these crumbled into a powder which could not be ingested. A firmer pellet was made by coating the stock pellets with crystalline cholesterol dissolved in ether.

Rabbits may contract coccidiosis if the drinking water becomes contaminated with faecal matter. Water was supplied from hanging bottles with metal drinking spouts. Bottles, feed containers, and cages were sterilized by steam once a week. No insecticide sprays were used. Removable pans beneath the wire mesh floor were washed with a disinfectant solution of substituted dimethylbenzyl ammonium chloride (Hytron).

Ergometrine Test.—Serial ergometrine tests on cholesterol-fed and control animals were done after 6 to 8 weeks on the diets and thereafter usually at weekly intervals until the test became positive. The general procedure has been described (Rinzler *et al.*, 1955, 1956). Rabbits were anaesthetized with pentobarbitone sodium (Nembutal Sodium Veterinary Abbott) 15 mg./kg. intravenously, or 10 mg./kg. in some atherosclerotic rabbits. The corrosive action of this alkaline solution caused sclerosis of ear veins and created technical difficulties on repeated testing until we reduced the *p*H slightly by adding dilute hydrochloric acid just before injection and also diluted the dose with an equal part of 0.85% sodium chloride.

The general condition of the cholesterol-fed rabbits was usually good, although they were sluggish in behaviour and tended to lose weight gradually after the initial period of a few weeks on the diet (Rinzler *et al.*, 1956). Thus, when placed on the diet, the average weight of the 29 cholesterol-fed animals was 1.87 kg. (1.47 to 2.29 kg.) and 1.67 kg. (1.07 to 2.73 kg.) when sacrificed for coronary perfusion. In contrast, the average weight of the 32 control rabbits initially was 1.76 kg. (1.27 to 2.47 kg.), and 1.93 kg. (1.19 to 2.63 kg.) when the experiment was terminated.

Control electrocardiograms were taken before, and 5 and 10 min. after, pentobarbitone injection. Lead II and a precordial lead approximating to V_4 were taken. Ergometrine maleate 0.05 mg./kg. was injected intravenously, and leads II and V_4 were repeated after 1, 3, 5, 7 and 10 min. or longer, depending on the duration of the effect. Depression of the S-T segment, if it occurred, was usually most marked 3 to 5 min. after injection, and as a rule lasted 15 to 30 min. but in some instances 1 to 2 hr. The ergometrine test is positive if the S-T segment is depressed transiently by 0.5 mm. or more below the isoelectric level.

One or two days after the ergometrine test became positive, the atherosclerotic heart was isolated and the coronary circulation perfused. In control rabbits which were fed on the stock diet for approximately equal periods of time, the heart was similarly isolated and perfused.

Perfusion. — A modification of the basic Langendorff perfusion apparatus (Anderson type) was used; the modified apparatus has been described by Garb, Penna, and Scriabine (1955), and is shown in Fig. 1. For the operation, rabbits were anaesthetized with pentobarbitone sodium 30 mg./kg. intraperitoneally. We followed Garb's surgical technique for isolating the heart, with minor modifications. In the atherosclerotic rabbit, care was needed not to perforate the often rigid and fragile aorta when the cannula was inserted. The heart was suspended in air by the cannula and amplitude of contraction was recorded from the apex of the heart. Perfusion with oxygenated Ringer-Locke solution (NaCl 7.0 g./l.; $NaHCO_3$ 2.1 g./l.; KCl 0.42 g./l.; $CaCl_2$ 0.21 g./l.; $MgCl_2$ 0.2 g./l.; and dextrose 1.8 g./l.; *p*H 7.2 to 7.4 at 37°) at a pressure of 52 cm. of water was started as soon as the cannula was connected with the perfusion system. The aortic valve remained closed at this perfusion pressure, and the solution flowed into the coronary arteries. The perfusate dripped from the pulmonary artery and was not recirculated. Water at 37° was circulated between the double walls of the glass chamber. The heart rate and amplitude of contraction were read from an ink writing kymograph. The rate of coronary flow was measured by a flowmeter with a glass ball (Emil Greiner Co.); readings on an arbitrary scale were converted to ml./min. by reference to the calibration curve of the flowmeter for water. In analysing the data, diastolic values were used for coronary flow.

It usually required about 15 min. for stabilization of baselines after starting the perfusion with Ringer-Locke solution. While this was continuously perfused, drugs were injected into the system close to the heart by means of a syringe attached to the upper end of a fine polyethylene catheter, the lower end of which opened within the bulb of the aortic cannula. The total time of injection was approximately 15 sec.

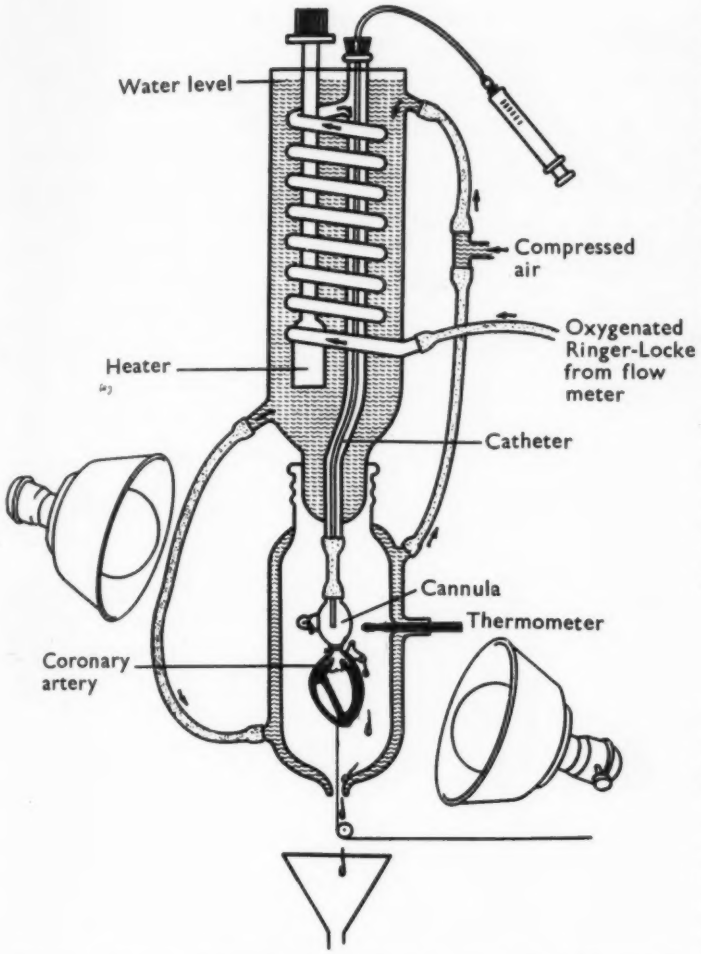

FIG. 1.—Diagram of the modified Langendorff perfusion apparatus.

for each dose. This method simulates the peak concentration of an intravenous injection in the intact animal. Each drug was injected in a volume of about 1 ml., and was followed at once by Ringer-Locke solution in a second syringe to make the volume 2 ml. and to wash the drug through the catheter. The capacity of the catheter was 0.3 ml. Control injections of 2 ml. of Ringer-Locke solution were made during every experiment. The effects of drugs were observed for at least 4 min., and longer if the effects lasted longer. Ringer-Locke solution alone was then perfused for 4 to 5 min. before the next dose or drug was given, except when ergometrine was injected while vasopressin acted. Continuous records were taken throughout the experiment, which usually lasted from 2 to 3 hr.

Drugs.—The drugs perfused in this study were: ergometrine (U.S.P. ergonovine maleate); purified arginine-vasopressin free of oxytocin, supplied by Dr. Vincent du Vigneaud as a solution (0.8 u./ml.) recently assayed by the blood pressure effect in the rat and without any preservative; and nitroglycerin, as 0.65 mg. hypodermic tablets dissolved in Ringer-Locke solution just before injection. In preliminary experiments, we determined the approximate threshold and maximal doses of these drugs for the isolated rabbit heart.

Pathology.—After perfusion was ended, the heart was fixed in formalin. For pathological study, cross-sections at 3 levels (base, centre, and apex) were stained with haematoxylin-eosin, Weigert (for elastin) and oil-red-O (for fat). In the ergometrine-positive rabbits with atherosclerosis of the small coronary arteries, the arterial changes were roughly graded as follows: 1+ subintimal foam cells without encroachment on the lumen of any artery sectioned; 2+ subintimal plaques extending partially into the arterial lumen; and 3+ obliteration of the lumen by subintimal atheroma in one or more arteries.

RESULTS

A total of 29 atherosclerotic hearts of cholesterol-fed, ergometrine-positive rabbits and 32 normal hearts of ergometrine-negative control rabbits were perfused with the various drugs.

Pathological Changes.—Most (96%) of the ergometrine-positive rabbit hearts showed advanced (2+ to 3+) intimal atherosclerosis of the small intramural arteries (Figs. 2 and 3); one heart showed 1+ atherosclerosis. The larger arteries seldom showed such changes. In the ergometrine-negative control hearts, atherosclerotic changes in the coronary arteries were not seen.

After perfusion, in both groups the myocardium appeared oedematous with separation of muscle bundles. However, in the non-perfused hearts of similar ergometrine-positive cholesterol-fed rabbits (Rinzler *et al.*, 1955, 1956), the myocardium showed diffuse fatty infiltration, spotty hyaline degeneration, and occasionally the fibrosis of old infarction (Fig. 3). Fragmentation of the elastic membrane beneath the subintimal plaques was also evident at many sites (Fig. 2).

Basal State of Isolated Heart.—Initially and before any drug, the coronary flow, heart rate, and amplitude of contraction were different in the normal and atherosclerotic hearts (Table I). In the atherosclerotic hearts, the average rate of coronary flow was about 4 ml./min. (24%)

FIG. 2.—Photomicrograph of a small coronary artery showing lumen partially occluded by subintimal plaques (2+ atherosclerosis) and fragmentation of underlying elastic membrane at margins of plaques, as indicated by arrows (Weigert stain). In this ergometrine-positive rabbit (11 weeks on cholesterol diet), the heart was sectioned immediately after death and was not perfused.

greater, the average heart rate was 25 beats/min. (17%) slower, and the average amplitude of contraction was 6 mm. (19%) less than in the normal hearts. No correlation was evident between basal coronary flow and heart weight, which ranged from 6.7 to 12.2 g. for 9 atherosclerotic, and from 6.6 to 11.6 g. for 23 normal hearts.

To determine whether the higher level of basal coronary flow in the atherosclerotic heart showed a correlation with the maximal capacity of the coronary bed to dilate, nitroglycerin in graded doses was injected in 8 experiments on atherosclerotic hearts, and, for comparison, in 7 experiments on normal hearts. Fig. 4 illustrates the typical effects of a maximal dose of nitroglycerin

TABLE I

INITIAL STATE OF ISOLATED PERFUSED HEART OF RABBIT

Figures in parenthesis indicate range.

Group	Total Hearts (No. of Expts.)	Coronary Flow (ml./min.)	Heart Rate (Beats/min.)	Amplitude of Contraction (mm.)
Normal	32	15·7 (9–28)	151 (98–200)	33·0 (7–79)
Atherosclerotic	29	19·5 (8–32)	126 (36–196)	26·3 (6–52)
	t test	$p<0·01$	$p<0·01$	$p<0·001$

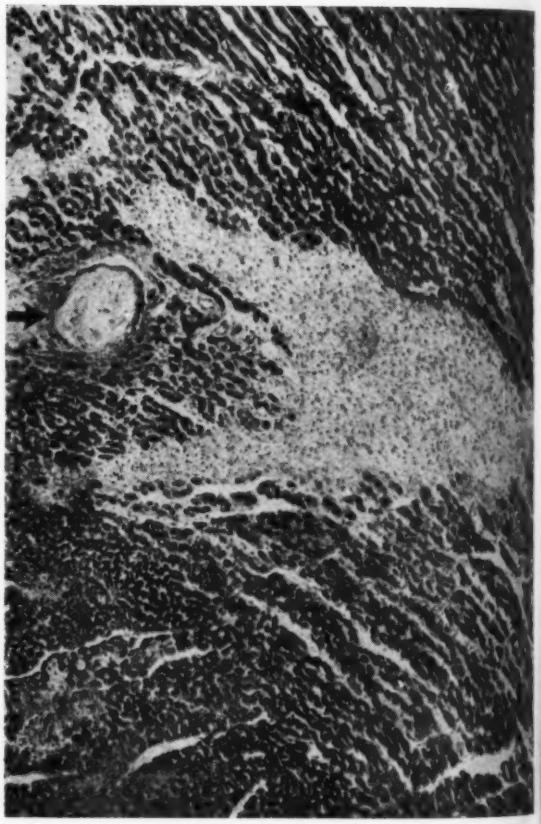

FIG. 3.—Photomicrograph of an old myocardial infarct with adjacent occluded small coronary artery (3+ atherosclerosis) at arrow (Masson stain). The heart was perfused before it was sectioned (rabbit 18 weeks on cholesterol diet).

in a normal and an atherosclerotic heart. In both, a marked increase in coronary flow occurred; in the normal, flow rose to 33 ml./min., and, in the atherosclerotic heart, to 29 ml./min., with little change in heart rate and slight reduction in amplitude of contraction. The highest level of coronary flow achieved with the maximal dose of nitroglycerin in the 7 normal hearts averaged 28.8 ml./min., and for the 8 atherosclerotic hearts 25 ml./min., a difference of 3.8 ml./min., or 13% higher in the normal group. In these small groups of animals, this difference was not statistically significant.

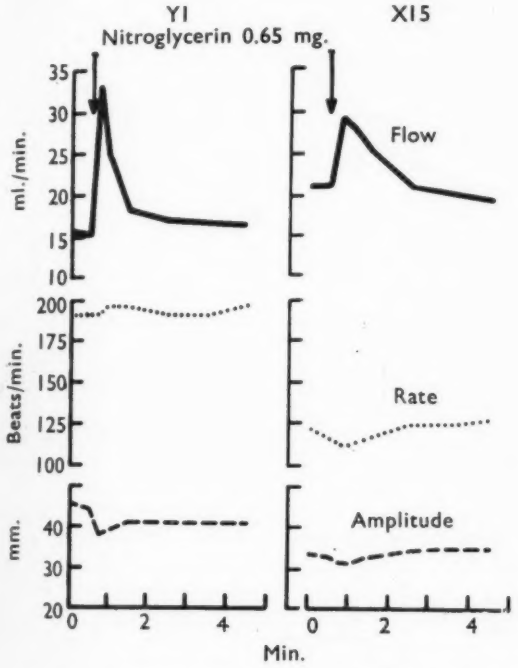

FIG. 4.—Response of perfused *normal* heart (Rabbit Y_1 at left) and *atherosclerotic* heart (Rabbit X_{15} at right) to a maximal dose of nitroglycerin. Note higher basal coronary flow in the atherosclerotic heart, but similar drug effects in both: increased flow and reduced amplitude of contraction with minor changes in heart rate.

Ergometrine.—Effects of graded doses of ergometrine (0.1 to 2.0 mg.) were studied in 15 atherosclerotic and 14 normal hearts; the average changes in coronary flow, heart rate, and contractile amplitude are shown in Fig. 5, and illustrative individual experiments on normal and atherosclerotic hearts in Fig. 6. Maximal changes usually occurred within 1 min. after injection of the dose, with a return to the control level within 2 min. In any given heart duplicate tests with the same doses gave results which agreed well (Fig. 7A). For example, in 25 comparisons with 61 doses of ergometrine on 13 hearts the mean reduction in coronary flow was 20.1% and the average difference from the mean 2.8% (S.E. ± 0.35).

Coronary flow was the only parameter that showed a significant difference in the response of the normal and atherosclerotic heart to ergometrine. Thus (Table II), after doses of 0.2 to 0.4 mg. in 47 tests on normal hearts, the average decrease in coronary flow was 8% as compared with 22% in 50 tests on atherosclerotic hearts. The greatest decrease in flow observed in any normal heart was 52%, whereas in the atherosclerotic group 3 hearts showed a decrease of 97%, 98%, and 100%, respectively. Furthermore, in the dosage range of 0.2 to 0.4 mg. ergometrine sometimes caused a significant increase (15% to 55%) in coronary flow in the 14 normal hearts, whereas in none of the 15 atherosclerotic hearts was there an increase in flow greater than 8%, which is comparable to the occasional effect of a control injection of Ringer-Locke solution.

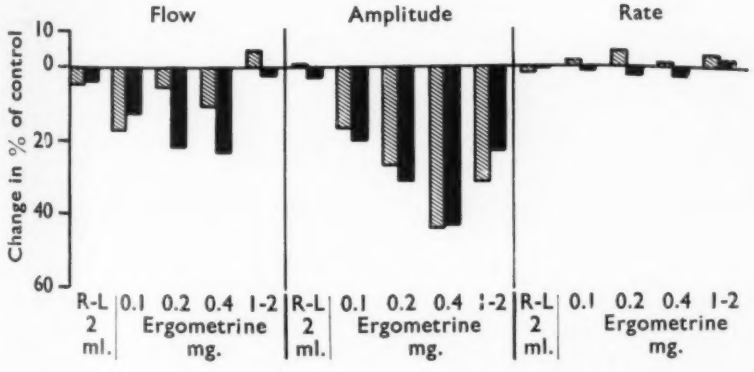

FIG. 5.—Average effects of graded doses of ergometrine on coronary flow, amplitude of contraction and heart rate in 14 *normal* (cross-hatched columns) and 15 *atherosclerotic* (black columns) perfused rabbit hearts. A control dose of Ringer-Locke 2 ml. was injected initially.

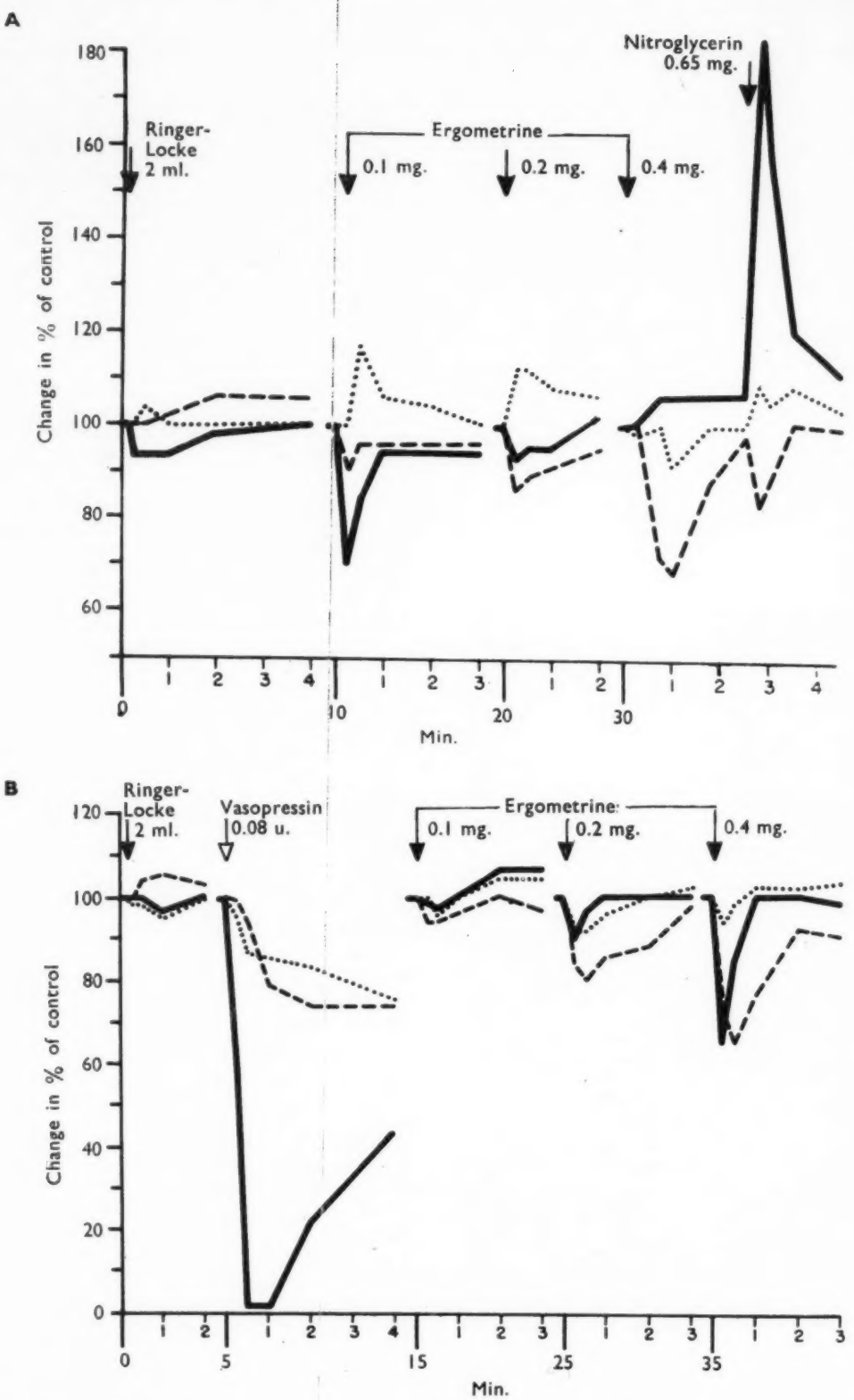

FIG. 6.—Illustrative experiments showing graded responses of flow ——, rate . . . . , and amplitude – – –, to 0.1 mg., 0.2 mg., and 0.4 mg. of ergometrine in (A) *normal* and (B) *atherosclerotic* hearts. Note also marked dilator effect of nitroglycerin after ergometrine in (A), and profound constrictor effect of vasopressin in (B).

Fewer tests were conducted with the smaller (0.1 mg.) and larger (1 mg. and 2 mg.) doses of ergometrine, and with these doses differences in the response of coronary flow in the normal and atherosclerotic groups were not statistically significant. However, for the small dose (0.1 mg.) in 19 tests on normal hearts, there was an average decrease of 17% in coronary flow, and in 20 tests on atherosclerotic hearts an average decrease of only 12%. With the largest doses (1 mg. and 2 mg.) the trend was toward a reversal of the vasoconstrictor effect; thus, in 11 tests on normal hearts coronary flow increased 6%, whereas in the atherosclerotic hearts it decreased only 2% (Fig. 5).

Amplitude of contraction decreased after all doses of ergometrine, and to about the same extent in normal and atherosclerotic hearts (Fig. 5, Table III). The % change was similar in both groups even though the average control amplitude for the atherosclerotic hearts was approximately one-half that for normal hearts before each dose of ergometrine. For example, after the 0.4 mg. dose the average decrease in amplitude in normal hearts was 44% from a level of 17.6 mm., and 43% from a level of 9.3 mm. in atherosclerotic hearts.

Graded responses in amplitude of contraction were obtained for doses of 0.1 mg., 0.2 mg., and 0.4 mg. of ergometrine. The average decrease for

TABLE II

COMPARISON OF EFFECTS OF ERGOMETRINE AND VASOPRESSIN ON CORONARY FLOW

Ergometrine effects were studied in 14 normal and 15 atherosclerotic hearts. Vasopressin effects were studied in 9 normal and 9 atherosclerotic hearts. Maximal change within 1 min. after injection is expressed in % of control (pre-injection) value. When duplicate tests were done, the average for each heart was used.

Coronary Arteries	Ringer-Locke 2 ml.		Ergometrine 0·2 to 0·4 mg.		Vasopressin 0·08 u.	
	Control Flow	Maximal Change	Control Flow	Maximal Change	Control Flow	Maximal Change
	ml./min.	%	ml./min.	%	ml./min.	%
Normal	15·2	−4·5	13·0	−8·1	12·6	−90·4
Atherosclerotic	21·0	−3·9	17·6	−22·0	17·5	−82·3
t test	$p>0·8$		$p<0·05$		$p>0·8$	

TABLE III

EFFECT OF GRADED DOSES OF ERGOMETRINE ON CARDIAC CONTRACTION AND RATE IN 14 NORMAL AND 15 ATHEROSCLEROTIC RABBIT HEARTS

Maximal change within 1 min. after injection is expressed in % of control (pre-injection) value. When duplicate tests were done, the average for each heart was used.

Drug and Dose	Amplitude of Contraction				Heart Rate			
	Control Level		Maximal Change		Control Level		Maximal Change	
	Normal	Athero.	Normal	Athero.	Normal	Athero.	Normal	Athero.
	mm.	mm.	%	%	Beats/min.	Beats/min.	%	%
Ringer-Locke 2 ml.	21·3	15·3	−0·1	−2·5	138	113	−1·5	−0·4
Ergometrine 0·1 mg. ..	24·6	13·9	−16·4	−19·9	141	115	+1·6	−1·8
0·2 ,, ..	21·4	11·6	−26·3	−30·3	132	113	+4·5	−2·5
0·4 ,, ..	17·6	9·3	−43·6	−42·9	150	115	+0·9	−2·8
1–2 ,, ..	16·7	8·7	−13·1	−22·6	138	114	+3·5	+2·1

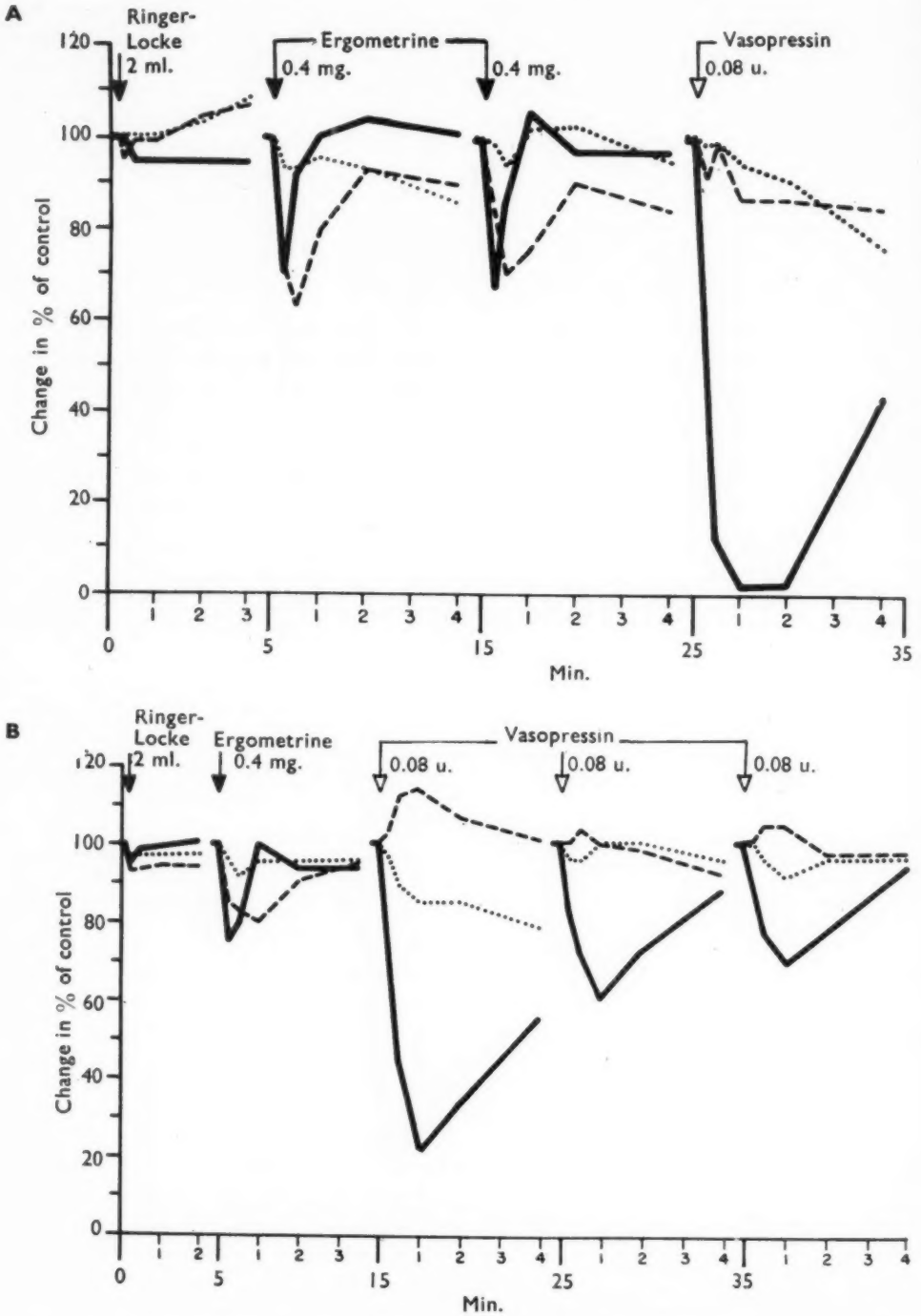

FIG. 7.—*Atherosclerotic* hearts. Flow ——, rate ...., and amplitude – – –. (A) Note reproducible effects of duplicate injections of ergometrine on all three parameters; also typical profound effect on flow of an initial dose of vasopressin, given after two doses of ergometrine. (B) Note limited reduction in flow after ergometrine as compared with initial dose of vasopressin, and tachyphylaxis to vasopressin when same dose was repeated three times.

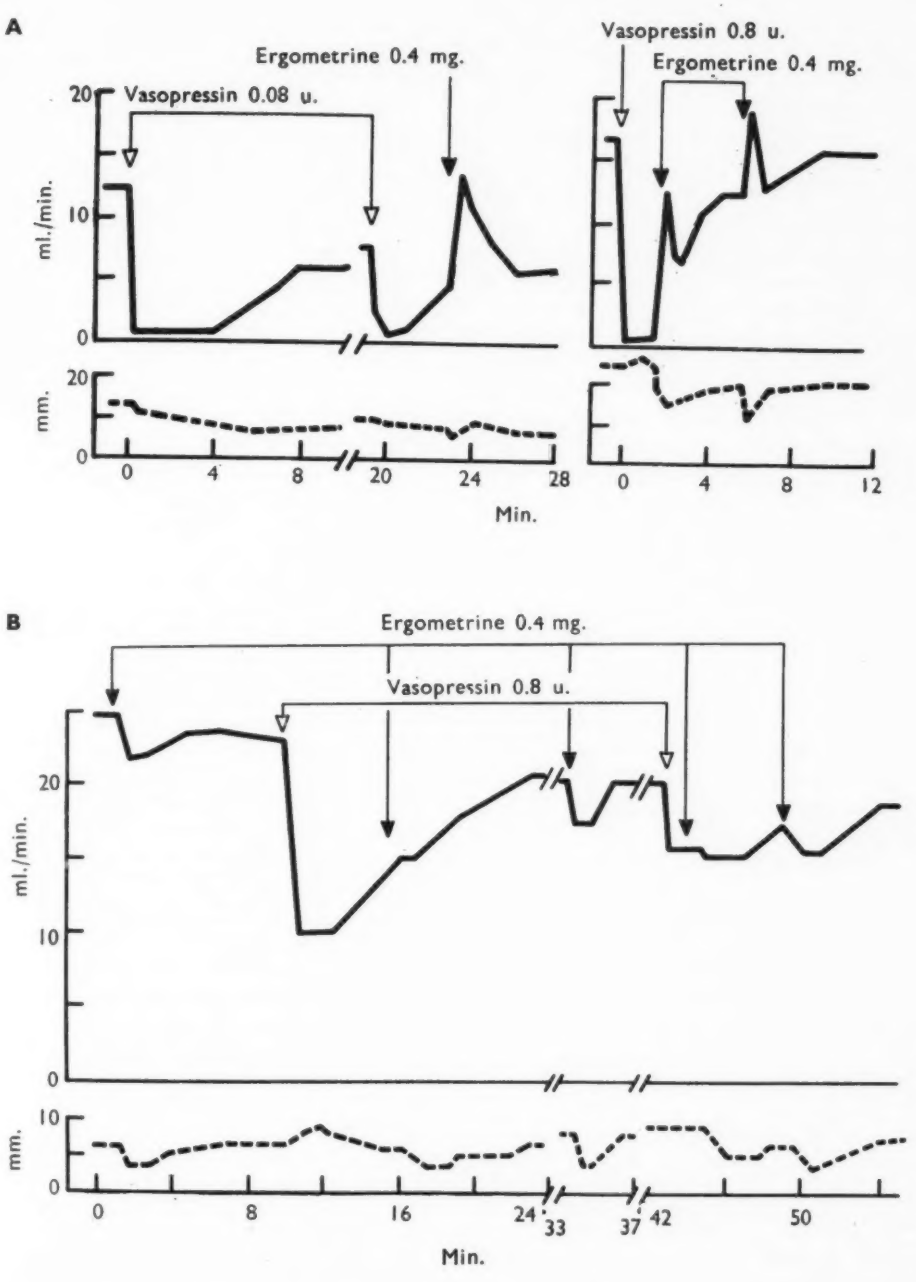

FIG. 8.—Responses of coronary flow —— and of amplitude – – –. (A) *Normal* hearts. Note coronary constrictor and negative inotropic effects of three doses of ergometrine (0.4 mg.) given during different phases of vasopressin action. (B) *Atherosclerotic* heart. Note coronary constrictor and negative inotropic effect of three doses of ergometrine (0.4 mg.) given during similar phases of vasopressin action. For comparison, control doses of ergometrine were given before and after the first dose of vasopressin. Heart rate was essentially unchanged in both *normal* and *atherosclerotic* hearts, and is not charted.

these doses in normals was −16.4%, −26.3%, and −43.6%, and in the atherosclerotic hearts, −19.9%, −30.3%, and −42.9%, respectively. However, with the larger doses (1 mg. and 2 mg.) the negative inotropic effect was less intense, namely, −13.1% and −22.6% in the respective groups (Table III).

The heart rate showed no consistent change after ergometrine (Fig. 5).

Vasopressin.—All hearts, normal and atherosclerotic, that were injected with ergometrine were tested with vasopressin; the dose of vasopressin ranged from 0.008 to 0.8 units. The effects of vasopressin showed striking differences from those of ergometrine.

Coronary flow was more profoundly reduced by the initial dose of vasopressin than by ergometrine both in normal and atherosclerotic hearts (Table II, Figs. 6B, 7 and 8). Thus, 0.08 units of vasopressin, maximal dose, decreased the average coronary flow by 90% in 9 normal hearts, and by 82% in 9 atherosclerotic hearts; this difference was not statistically significant (Table II).

Unlike ergometrine, successive identical doses of vasopressin yielded diminishing effects (Fig. 7B). This tachyphylaxis to vasopressin was observed for both atherosclerotic and normal hearts. The typical profound reduction in flow after the first dose of vasopressin was not blocked if ergometrine was given first (Fig. 7), nor did a prior dose of vasopressin modify the effect of ergometrine given after the vasopressin effects had finished (Figs. 6B and 8B). The reduction in coronary flow by vasopressin could be momentarily reversed by nitroglycerin.

Amplitude of contraction after vasopressin sometimes increased initially, but usually decreased if the reduction in coronary flow was profound and prolonged (Figs. 6B, 7 and 8A). In this respect, no essential difference was observed between atherosclerotic and normal hearts.

Heart rate after vasopressin usually decreased in both atherosclerotic and normal hearts (Figs. 6B, 7 and 8). Both contractile amplitude and heart rate returned to control levels more slowly than did coronary flow.

Ergometrine During the Action of Vasopressin.—When ergometrine (0.4 mg.) was given about 1 min. after vasopressin, when the coronary flow was most reduced, ergometrine caused no change in flow in either normal or atherosclerotic hearts (Table IV). However, when ergometrine was given while the vasoconstrictor action of vasopressin was wearing off, usually in 1.5 to 2 min. after vasopressin injection, the effect of ergometrine on coronary flow differed significantly for normal and atherosclerotic hearts (Table IV). In the normals, a transient rise in flow after ergometrine (8 doses) was uniformly superimposed on the rising slope of recovery from the vasopressin reduction (Fig. 8A). On the other hand, in the atherosclerotic hearts, only 1 of 8 similar doses of ergometrine caused any increase in flow; 6 of the doses caused a transient reversal of the vasopressin recovery curve, and in the remaining 1 the rising slope was temporarily flattened (Fig. 8B).

TABLE IV

EFFECT OF ERGOMETRINE ON CORONARY FLOW WHEN INJECTED DURING VASOPRESSIN CONSTRICTION IN 7 NORMAL AND 5 ATHEROSCLEROTIC HEARTS

Ergometrine given early, 0·25 to 1 min. after vasopressin, in column at right; other doses given later, 1·5 to 23 min. after vasopressin, during rising flow.

Heart	Total Doses Ergometrine (0·4 mg.)	Ergometrine-induced Flow Change during Vasopressin Action		
		Increased	Decreased	Unchanged
	No.	No.	No.	No.
Normal	10	8	0	2
Atherosclerotic	12	1	7	4

In these experiments, the dose of vasopressin ranged from 0.008 to 0.8 units, and showed no correlation with the direction of the ergometrine effect on flow during vasopressin action.

When ergometrine was given while vasopressin acted, the usual reduction in amplitude was seen regardless of whether coronary flow increased or decreased (Fig. 8). The heart rate showed negligible changes.

DISCUSSION

Basal State of Isolated Heart.—The finding that the atherosclerotic heart in its initial state, before any drugs were perfused, had a higher coronary flow than normal was unexpected. Since these observations were made, however, it has been reported (Gorlin, Brachfeld, Macleod, and Bopp, 1959) that in man the resting coronary flow determined by cardiac catheterization likewise is significantly higher in patients with coronary insufficiency (effort angina) than in normal subjects.

Wegria (1951) showed in the normal dog that blood flow is determined by the diastolic pressure at the coronary ostia and by the resistance of the coronary bed. Since the aortic (perfusion) pressure is constant in our isolated rabbit heart preparation, the observed differences in basal coronary flow must depend on differences in coronary resistance. Theoretical factors which might alter coronary resistance of the atherosclerotic heart in the direction of an increased basal coronary flow include: (1) an increase in collateral circulation during the course of atherogenesis with enlargement of the total coronary bed; (2) increased energy expenditure of the heart owing to impaired myocardial efficiency; (3) a hyperaemic response to localized areas of anoxic myocardium, mediated by the intrinsic neurohumoral mechanisms of the heart; (4) yielding of the atherosclerotic vascular wall owing to fragmentation of the subintimal elastic membrane in the small coronary arteries; and (5) weakening of the extravascular support of the coronary bed owing to extensive damage to the myocardium.

In considering mechanical factors in the rate of coronary flow, Duguid and Robertson (1957) point out that it is the large coronary arteries which receive most of the impact of ventricular systole, and thus their functional state rather than the resistance of the terminal arterial bed largely determines the maximal capacity of the coronary bed to dilate. In the atherosclerotic rabbit heart, the large coronary arteries are relatively free of atherosclerotic plaques even when the intramural vessels are extensively occluded. It is not surprising, therefore, that the coronary dilator effect of nitroglycerin in the atherosclerotic rabbit heart is only moderately or little impaired. At the same time, lowered resistance in the smaller arteries would be compatible with a higher basal coronary flow in the atherosclerotic group.

The known depletion of myocardial catecholamines in coronary artery disease (Raab and Gigee, 1955) would explain the diminished amplitude and slower rate of contraction observed initially in the atherosclerotic, as compared with the normal isolated rabbit heart. Lee and Shideman (1959) have demonstrated in cats that a reduction in myocardial catecholamines by sympathectomy or reserpine does indeed cause diminished contractility of isolated cardiac (papillary) muscle.

Differences in Ergometrine Effects on Normal and Atherosclerotic Hearts. — Mechanisms by which ergometrine causes electrocardiographic changes typical of coronary insufficiency in man and the intact rabbit with latent coronary artery disease, but not in normal persons or animals, are suggested by the results of this investigation.

The consistent and considerable negative inotropic effect of ergometrine, although equal in normal and atherosclerotic isolated rabbit hearts, may nevertheless play a role in the production of electrocardiographic changes in the intact atherosclerotic animal. In the isolated heart, ergometrine causes a similar percentage decrease in contractile amplitude for both groups, but the absolute level to which the amplitude falls is much lower for atherosclerotic hearts since the amplitude is initially less, only half that of the normals. If this reduction represents a deleterious effect on heart muscle with impaired oxygen utilization, it may well contribute to the S-T segment depression induced by ergometrine in the intact animal, especially when a reduction in coronary flow is superimposed.

On the average, the quantitative effect of ergometrine on coronary flow differs significantly in the normal and atherosclerotic groups at the dose range of 0.2 to 0.4 mg.; in the normals the reduction in flow was 8% as compared with 22% in the atherosclerotic. For the smaller and larger doses of ergometrine that we employed, the numbers of experiments were too small for differences to be statistically significant.

In the atherosclerotic heart, ergometrine quite uniformly causes a transient decrease in coronary flow. In the normal heart, the response is more variable; increases as well as decreases in flow occurred. The coronary dilator action of ergometrine in normal hearts is most consistently seen when the coronary bed is partially constricted by vasopressin. In contrast, under these conditions ergometrine has a predominantly constrictor effect in the atherosclerotic heart.

A reduction in coronary flow of about 25% has been shown not to cause electrocardiographic changes in the normal intact animal (Wegria, Segers, Keating, and Ward, 1949). On the other hand, in the presence of occlusive disease of the small coronary arteries and myocardial damage, a flow reduction of this magnitude may be critical and so contribute to anoxic changes in the electrocardiogram. The delay of several min. in the appearance of S-T segment depression after intravenous ergometrine in the intact atherosclerotic rabbit and in the patient with effort angina suggests that this effect depends on myocardial ischaemia secondary to constriction of the diseased coronary arteries, rather than solely on a direct action of the drug on the myocardium.

In contrast to the reproducible effects of a given dose of ergometrine, vasopressin induces tachyphylaxis in the isolated heart. Furthermore, the effects of vasopressin are essentially similar for normal and atherosclerotic isolated rabbit hearts. These observations suggest fundamental differences in the mechanism of action of these two drugs on the heart. One may speculate that the effects of ergometrine on coronary flow are mediated at least in part through mobilization of catecholamines, whereas vasopressin acts more directly on receptors. When catecholamines are depleted during atherogenesis, a direct action of ergometrine on receptors may be unmasked.

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THE EFFECT OF TEMPERATURE ON THE DIRECT MUSCLE TWITCH RESPONSE AND THE ACTION OF DRUGS ON THE ISOLATED DENERVATED RAT DIAPHRAGM

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The effect of temperature on the direct twitch response of the normal and denervated rat diaphragm, and the sensitivity of the contractural response of the denervated muscle to acetylcholine, suxamethonium, decamethonium, and potassium salts, was studied. Cooling the normal diaphragm from 39 to 20° increased the size of the direct twitch, but below this temperature the twitch response of the muscle was progressively depressed. The denervated muscle twitch was, by contrast, maximal at 39° and decreased as the temperature was lowered. This property of the denervated muscle first appeared about the third day after denervation. The contractural response of the denervated diaphragm to applied acetylcholine was enhanced by cooling, while the response to potassium salts was depressed. The depression of the contracture induced by potassium followed closely the depression of the direct muscle twitch of the denervated muscle over the same temperature range.

Previous work on the relation between temperature and the size of the direct muscle twitch response has indicated that generally the tension developed in a twitch is greater at low temperature. This has been found for the frog sartorius over temperature ranges from 0 to 20° (Hill, 1951), whereas studies on the rat triceps (Walker, 1949) have indicated that an 80 to 90% increase in the size of the indirect muscle twitch occurs on cooling from 37 to 24°. These findings have been confirmed by Doudoumopoulos and Chatfield (1959), who found that the twitch tension of the rat gastrocnemius was greatest between 26 and 18°. Work on the cat tibialis anterior and rat diaphragm muscles (Maclagan and Zaimis, 1957; Bigland, Goetzee, Maclagan, and Zaimis, 1958), however, indicates that in these muscles lowering of temperature decreases the size of the twitch response. The present studies were made to investigate whether both the normal and denervated rat diaphragm showed similar or dissimilar changes in the size of the direct twitch response when cooled.

Recently it has been shown that cooling markedly increases both the magnitude and duration of action of depolarizing blocking agents in cat muscle (Zaimis, 1958; Bigland *et al.*, 1958), whereas the blocking action of competitive agents is reduced by cooling (Holmes, Jenden, and Taylor, 1951). In view of these findings it seemed important to establish whether the contractural response produced by acetylcholine (Frank, Nothmann, and Hirsch-Kaufmann, 1922; Gasser and Dale, 1926; Dale and Gaddum, 1930; Knowlton and Hines, 1937; Brown, 1937) and potassium salts (Frank, Nothmann, and Guttmann, 1923; Gasser and Dale, 1926; Brown, 1937) in denervated mammalian muscle was materially affected by cooling.

METHODS

The experiments were made on the isolated rat diaphragm preparation (Bülbring, 1946), denervated some days previously by removal of the right phrenic nerve under ether anaesthesia. The muscle was suspended in a bath of 50 ml. capacity and bathed with Krebs solution aerated with 95% oxygen and 5% carbon dioxide. Stimulation of 3 milliseconds duration at 120 volts was delivered at 5 per min. Twitch responses to electrical stimulation, together with contractural responses to various drugs, were recorded by means of a semi-isometric lever on a smoked drum. The doses of drugs are expressed as their final bath concentrations.

In the experiments on the effect of temperature on the direct twitch response of the denervated muscle, the normal diaphragm of the opposite side was used as a control.

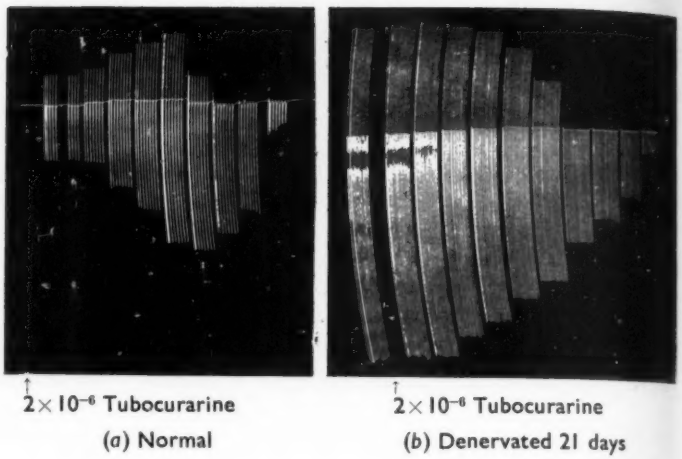

FIG. 1.—The effect of cooling on the direct twitch response of (*a*) a normal rat diaphragm, and (*b*) a 21-day denervated diaphragm. Both muscles were suspended in Krebs solution containing 2×10^{-6} tubocurarine. The normal muscle was cooled in 4° steps (left to right) from 39° to 3°. The denervated muscle was cooled in 3° steps from 39° to 12°.

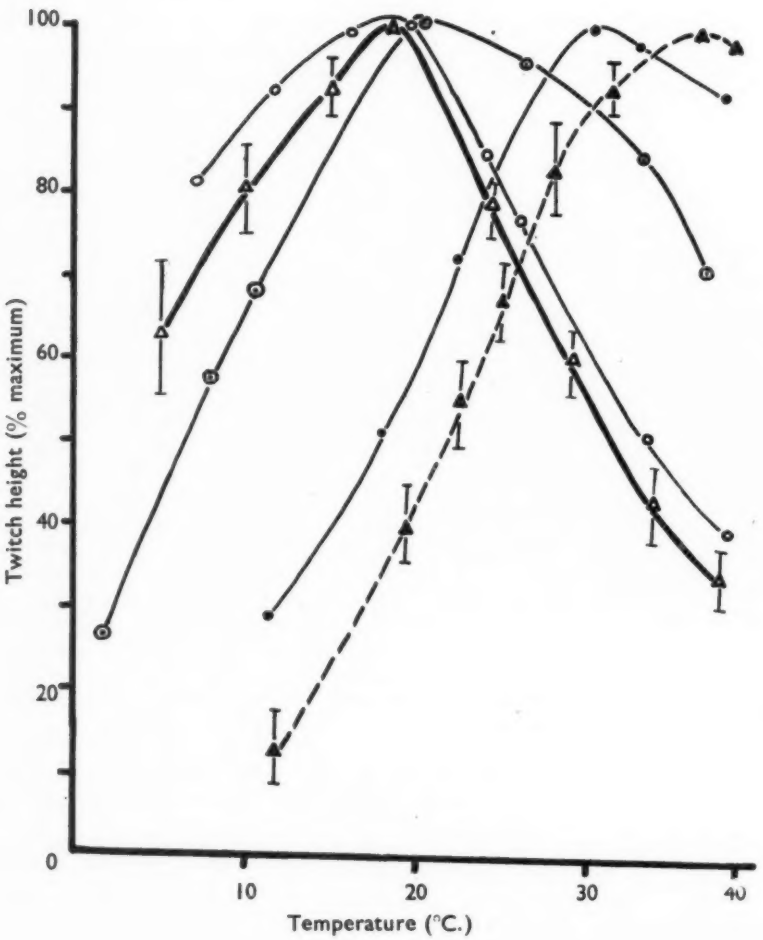

FIG. 2.—The relation between the direct twitch response and temperature for normal and denervated diaphragms in the presence of tubocurarine. The curves represent: (△——△) the mean for normal muscles, (▲- - -▲) the mean for muscles denervated from 3 to 28 days, (O——O) 1-day denervated muscle, (⊙——⊙) 2-day denervated muscle, and (●——●) 3-day denervated muscle.

RESULTS

The Effect of Temperature on the Size of the Direct Twitch Response of the Normal and Denervated Diaphragm

In all experiments on both normal and denervated muscles a concentration of 2×10^{-6} tubocurarine was maintained to abolish any neurally mediated effects, thereby ensuring that the twitch responses recorded were direct muscle responses.

Fig. 1 shows records from (*a*) a normal muscle, and (*b*) a muscle denervated for 21 days. The size of the direct twitch response of the curarized denervated muscle at 39° was much greater than that of the curarized normal muscle at the same temperature. As the bath temperature was lowered the size of the direct twitch response of the normal muscle increased down to a temperature of approximately 20°, while below this temperature the twitch response became sluggish (Li, 1958). In marked contrast to the normal diaphragm, the direct twitch response of the denervated muscle underwent a progressive decline as the temperature was lowered; also the twitch itself became sluggish in form at a higher temperature in the denervated muscle than in the normal muscle.

The results of a large number of experiments on normal and denervated diaphragms are graphically presented in Fig. 2. The mean curves which relate the size of the direct muscle twitch to temperature for the normal and denervated muscles (from 3 to 28 days) are significantly different. The normal diaphragms showed their greatest twitch responses at nearly 20°, whereas the denervated muscles gave their greatest twitch responses around 39°. The curve for the muscle denervated for 1 day shows but little departure from the normal, unlike that for the muscle denervated 3 days which corresponds closely to the mean curve for muscle denervated from 3 to 28 days. These findings therefore show that denervation brings about a characteristic change in the relation between the size of the direct muscle twitch response and temperature; this change first appears about the second day after denervation and is complete soon after the third day.

The Effect of Temperature on the Contractural Response of the Denervated Diaphragm to Acetylcholine, Suxamethonium, and Decamethonium

Cooling the denervated muscle to 30, 20, and 10°, respectively, progressively increased the magnitude and duration of the contractural response which the standard (2×10^{-6}) dose of acetylcholine elicited (Fig. 3). Further, the threshold dose of acetylcholine which excited the denervated muscle at 39° was reduced by at least one log-dose on cooling to 20°. When the muscle was rewarmed the original sensitivity to acetylcholine was regained, indicating that the sensitization of the muscle to acetylcholine by cooling was a reversible process. Similar experiments carried out in the presence of 5×10^{-7} neostigmine have shown that the presence of an anti-cholinesterase in no way modifies the sensitization of the muscle to acetylcholine at low temperature, an observation which suggests that the hypersensitivity was not caused by the inactivation of the muscle cholinesterase by cooling. The contractural responses of the denervated diaphragm to acetylcholine were incompletely blocked by tubocurarine, 2×10^{-6}.

Suxamethonium and decamethonium also showed increased activity on the denervated diaphragm at low temperature. Generally, it was found that the degree of sensitization seen for

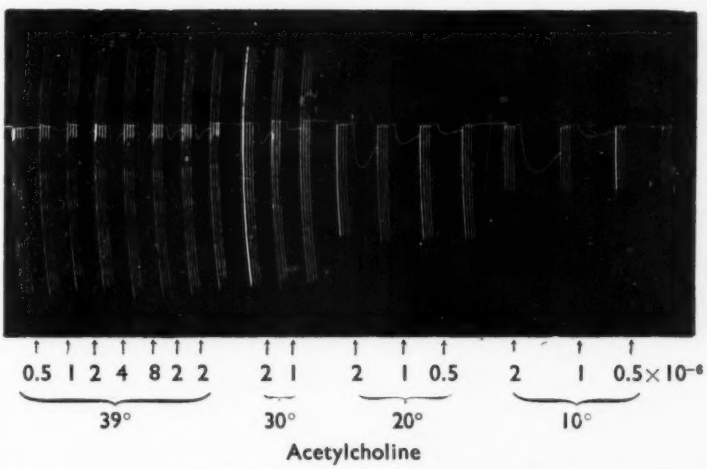

FIG. 3.—Contractural responses of the denervated diaphragm to acetylcholine are interposed between groups of direct twitch responses. On the left are contractures to doses of 1, 2, 4, and 8×10^{-6} acetylcholine. The test dose of 2×10^{-6} acetylcholine was then given to the muscle at 39°. Cooling the preparation to 30, 20, and 10° progressively enhanced the contractural response of the denervated muscle to the test dose of acetylcholine.

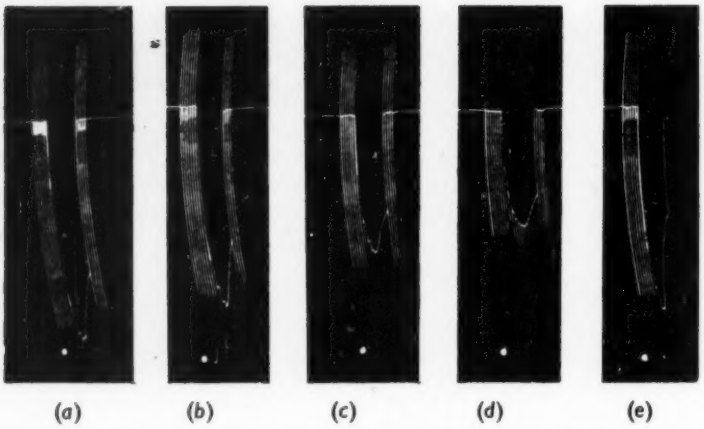

FIG. 4.—At (O) potassium chloride was added to the bath to give a concentration of 5×10^{-3}. Contractural responses of the same denervated diaphragm to 5×10^{-3} potassium chloride at (*a*) 38°, (*b*) 34°, (*c*) 30°, and (*d*) 22°. Rewarming the muscle to 39° (*e*) restored the original sensitivity of the preparation to the test dose of potassium chloride.

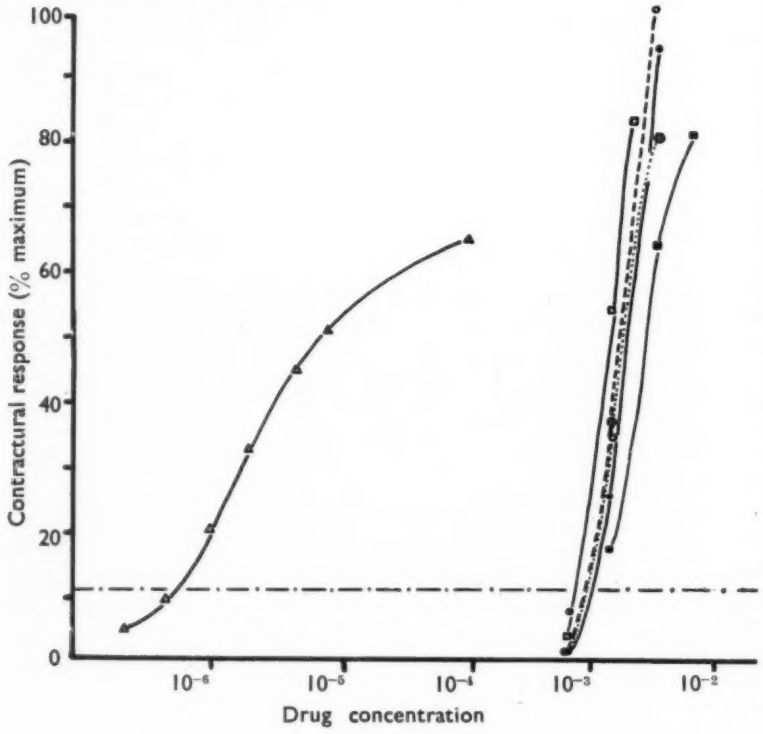

FIG. 5.—The dose-response relations for the contractures produced in the denervated rat diaphragm at 37° by acetylcholine (△——△), potassium chloride (●——●), potassium bromide (⊙ – – ⊙), potassium iodide (O – – O), potassium nitrate (□——□) and potassium sulphate (■——■). The contractural response elicited by a sucrose solution isosmotic with a solution of 5×10^{-3} potassium chloride is represented (—·—·).

suxamethonium was about the same as that found for acetylcholine. The sensitization seen with decamethonium was usually much smaller than that with acetylcholine.

The Effect of Temperature on the Contractural Response of the Denervated Diaphragm to Potassium Salts

Large doses of potassium salts (1×10^{-3}) were found to depress the electrical excitability of the denervated muscle, confirming the findings of Hajdu, Knox, and McDowall (1950). However, still larger doses, 5×10^{-3}, in addition to depressing the electrical excitability of the muscle also produced a typical contracture (Frank, Nothmann, and Guttmann, 1923; Gasser, 1930). These doses of potassium salts produced contractures which were attributed specifically to the presence of the potassium ion since the nature of the potassium salt used only slightly influenced the responses obtained. Control experiments showed that the effect was not caused by any effect of the potassium salts on the *p*H of the Krebs solution, or an osmotic action, since an osmotic solution of sucrose produced only a very small contracture (Fig. 5).

Cooling, instead of producing enhancement of the contractural response as was found to be the case with acetylcholine, suxamethonium, and, to a lesser extent, with decamethonium, depressed the contractural response to potassium chloride (Fig. 4). The depression of the contractural response to potassium salts on cooling paralleled the simultaneous depression of the twitch response.

The contractural responses of the denervated diaphragm to doses of 5×10^{-3} potassium chloride were unaffected by tubocurarine, 2×10^{-6}.

Discussion

Frog skeletal muscle is known to give a greater twitch at low temperature attributed to the temperature coefficient of the velocity of shortening being less than the temperature coefficient of relaxation (Hill, 1951). It is possible to account for some of the present findings in the normal rat diaphragm in this way. This might well be the reason for the increase in size of the twitch response of the normal diaphragm on cooling to 20°, but additional factors probably come into play when the muscle is cooled below 20° to cause the twitch response to gradually decline. Thus below 20° the oxygen consumption of the rat is decreased and at the same time there is a rise in the blood sugar and in the level of the plasma inorganic phosphate (Andjus and Smith, 1955). In addition to these findings, Li (1958) reported that the efficiency of the rat neuromuscular junction was greatest at temperatures above 15° while below this temperature changes in the size and duration of the muscle action potentials could be observed.

It would seem from Hill's (1951) work that, in the rat, denervation may, in effect, cause the temperature coefficient of the velocity of shortening to become greater than the temperature coefficient of relaxation, possibly by bringing about fundamental changes in the biochemical processes of the muscle related to contraction. Whatever the true explanation of these phenomena may be, it is of considerable interest that the appearance of these new properties of the denervated muscle, with respect to the change in its twitch response on cooling, should be on or about the third day after denervation, as it is at this time also that the denervated rat diaphragm is known to develop contractural properties to depolarizing drugs (Paterson, 1957). This would seem strong evidence for regarding the progressive depression of the twitch response of denervated muscle on cooling as a property *per se* of denervated muscle. Axelsson and Thesleff (1959) have recently drawn attention to the fact that denervated mammalian muscle will display increased sensitivity to humoral agents and other drugs only when there is a complete lack of connexion between the muscle and living nerve tissue. In support of this view, Jefferson, Phillips, and Necheles (1950), Jefferson, Ogawa, and Necheles (1958), and Jefferson, Ogawa, Toman, Scruggs, and Necheles (1959) have shown that the degenerative changes which normally appear in the diaphragm of the dog after phrenic nerve section can be prevented by either anastomosing the peripheral cut end of the phrenic to the other cut end of the same phrenic, or to the vagus nerve. These findings suggest that the degenerative changes appear in denervated mammalian muscle only when the trophic influence of a functional, or potentially functional, nerve supply has been lost. If this is so, the appearance of the characteristic twitch responses of the denervated rat diaphragm might well be associated with the loss of the trophic influence of the phrenic nerve innervation.

The probable explanation why cooling enhances the contractural responses elicited with acetylcholine and suxamethonium and yet depresses those produced by potassium salts is that these two groups of compounds produce contracture in denervated muscle by different mechanisms. Some evidence in favour of the dissimilar modes of action of acetylcholine and potassium ions has already been put forward in the literature. Brown and Feldberg (1936) found that 7×10^{-4} tubocurarine blocked the response of the perfused superior cervical ganglion to pre-ganglionic impulses and to applied acetylcholine but not to potassium chloride. Buchthal and Lindhard (1939) were also impressed by the fact that lizard muscle could still be stimulated with potassium chloride when it was refractory to nerve stimulation and applied acetylcholine. They suggested, on this basis, that acetylcholine and potassium chloride acted upon different regions of the muscle endplate. In agreement with these workers it was found in the present study that the contractural responses of the denervated diaphragm produced by acetylcholine were blocked by a dose of 2×10^{-6} tubocurarine whereas those produced by doses of 5×10^{-3} potassium chloride were completely unaffected by the tubocurarine. Examination of the log-dose curves for acetylcholine, potassium chloride, potassium bromide, potassium nitrate, and potassium sulphate for this preparation (Fig. 5) shows that the curve for acetylcholine is not parallel to those for the potassium salts which are grouped closely together, and are themselves parallel. Further, the graph indicates that acetylcholine can evoke a contracture from the denervated muscle in doses approximately 1,000 times smaller than those required for potassium salts (Brown, 1937).

The evidence presented above indicates that acetylcholine and related substances trigger a contracture in denervated mammalian muscle by combining with receptors which, according to Axelsson and Thesleff (1959), are situated diffusely over the whole of the outer surface of the muscle membrane. Potassium salts appear to produce contracture in the denervated muscle either by

acting directly on some other region of the former endplates or muscle membrane generally, or by an intracellular mechanism. An intracellular mechanism cannot be ruled out since massive doses of potassium salts have to be given before any contracture is seen, and potassium sulphate, the least permeable of the potassium salts used, was the weakest of the potassium salts in producing contracture in the denervated rat diaphragm.

The results suggest that the biochemical processes concerned with the production of a mechanical twitch after an electrical stimulus, together with those which produce a contracture in the denervated muscle on the application of potassium salts, are similarly depressed by cooling. That enhancement of the contractures produced by acetylcholine is seen on cooling may therefore be explained by specific sensitization of the muscle receptors by cooling.

I am greatly indebted to Professor G. Brownlee for his advice and criticism. The experiments were carried out during the tenure of a Medical Research Council Scholarship.

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THE EFFECT OF ENZYME INHIBITORS ON HISTAMINE CATABOLISM IN MAN

BY

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The catabolism of histamine was studied in three female psychiatric patients by analysis of the [^{14}C]-labelled metabolic products occurring in the urine after a subcutaneous injection of [^{14}C]histamine. Each patient was studied before and during treatment with aminoguanidine or iproniazid.

Without treatment the patients had a normal histamine catabolism. Aminoguanidine and iproniazid inhibited the oxidation of histamine to imidazoleacetic acid; iproniazid produced a 50% inhibition of the oxidation of methylhistamine to methylimidazoleacetic acid. After iproniazid a large proportion of the injected [C^{14}]histamine was excreted as methyl [^{14}C]histamine.

The catabolism of histamine in various species, including man, has been elucidated by Schayer and his co-workers by means of [^{14}C]-labelled histamine (for references see Schayer, 1959). The pathways for the breakdown of histamine have been studied in normal men (Schayer and Cooper, 1956) and in non-pregnant and pregnant women (Nilsson, Lindell, Schayer, and Westling, 1959). So far, there has been no study, in man, of the effect of substances which inhibit the enzymes involved in the catabolism of histamine. From animal experiments (Schayer, 1959) it is known that histaminase (diamine oxidase) can be inhibited very effectively by aminoguanidine in doses which appear to be non-toxic. The enzyme that methylates histamine cannot yet be effectively inhibited *in vivo*, but the oxidation of the methylated histamine is blocked in mice by iproniazid (Rothschild and Schayer, 1958).

Mitchell (1956) measured the urinary excretion of non-labelled histamine in humans before and after subcutaneous injection of histamine. Treatment by mouth with aminoguanidine in large doses, or with iproniazid, caused only small changes in the urinary histamine and in the percentage of injected histamine recovered from the urine. Mitchell therefore suggested that the enzymes concerned in histamine catabolism in man were either different from those in animals, or less sensitive to the inhibitory actions of aminoguanidine and iproniazid than could be expected from the animal experiments of Schayer.

In the present study the catabolism of [^{14}C]-labelled histamine was studied in three psychiatric patients before and during the administration of aminoguanidine or iproniazid. It was felt that such a study might give more information about the effectiveness of the inhibitors. Furthermore, iproniazid is now much used in psychiatry, and information about its various effects in humans is therefore of interest.

METHODS

Three female patients at Lillhagen's Mental Hospital were studied. The patients were:

A. Born in 1904. Schizophrenia-like condition since 1941. At present no symptoms of "active" disease, but dementia with periods of auditory hallucinations. Duodenal ulcer 1928 and 1931; rheumatoid arthritis 1938; erythema nodosum 1941. Since then somatically healthy. Menopause 1956. After 0.5 mg. histamine dihydrochloride subcutaneously, normal reactions (facial flush and tachycardia). Treatment with iproniazid gave no mental changes.

B. Born in 1908. Schizophrenia-like condition since 1936. Mental condition similar to that of patient A, with no signs of "active" disease. Auditory hallucinations. Somatically healthy. Low muscular "tone." Menopause 1954. Normal reaction to 0.5 mg. histamine. Treatment with iproniazid gave no mental changes.

C. Born in 1902. 1934 treated for acute encephalitis (diagnosis not quite certain). Between

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1934 and 1957 periods of depression. Chronic depression since August, 1957, that is, 1.5 years before given iproniazid. Paraesthesia in lower legs and feet, but neurologically normal. Otherwise somatically healthy. Menopause in 1934 in connexion with the encephalitis. Normal reaction to 0.5 mg. histamine. Significant mental improvement during treatment with iproniazid 50 mg. 3 times daily. Improvement maintained on half of this dosage. Five months after stopping the iproniazid treatment, the patient was given aminoguanidine by mouth in a dose of 50 mg. 4 times daily for 9 days, and then at twice this dosage for another 12 days. This treatment caused no mental improvement.

Iproniazid (" Marsilid," Roche) was given in doses of 50 mg. 3 times daily. Aminoguanidine sulphate (Eastman Organic Chemicals), dissolved in distilled water and sterilized by filtration, was injected subcutaneously in a dose of 0.1 mg./kg. (patient B), or 1.0 mg./kg. (patients A and C) at 3-hourly intervals.

[^{14}C]Histamine (The Radiochemical Centre, Amersham, England), dissolved in 0.9% saline containing 0.2% glucose and sterilized by filtration, was injected subcutaneously at about 8 a.m. The dose was 10.2 to 11.5 μg. histamine base, specific activity 42.7 μC/mg. base. Each patient received three injections of [^{14}C]histamine, in all about 1.5 μC, the first one before any treatment, the second one after 3 weeks of treatment with iproniazid, and the third one 3 to 6 months after stopping the iproniazid administration. Injections of aminoguanidine were begun 30 min. before the third injection of [^{14}C]-histamine and continued for 12 hr. in all 4 injections of aminoguanidine.

Patient C was given a fourth injection of [^{14}C]histamine, 2 months after the third one. The patient was not given any treatment during these 2 months. The fourth injection was given when the patient had taken aminoguanidine sulphate, 100 mg. 4 times daily by mouth for 7 days.

Urine was collected in 2 portions, 0 to 12 hr. and 12 to 24 hr. after the injection of [^{14}C]histamine. Great care was taken to obtain all urine from the patient during the 24 hr. after injection.

The analysis of the [^{14}C]-containing compounds in the urine was carried out as described earlier (Lindell and Schayer, 1958a; Nilsson *et al.*, 1959). The following substances were determined: histamine, methylhistamine [4-(2-aminoethyl)-1-methylimidazole], total imidazoleacetic acid (imidazol-4(5)-ylacetic acid), and methylimidazoleacetic acid (1-methyl-imidazol-4-ylacetic acid). Briefly the analytical procedure is as follows: a carrier amount (non-radioactive) of the substance to be determined is added to an aliquot of the urine specimen; a preliminary extraction is made (butanol extraction for histamine, chloroform extraction for methylhistamine, and absorption on an anion-exchange resin for the acids); the compounds are crystallized as picrates, and the picrates of histamine and imidazoleacetic acid are converted to derivatives of *p*-iodophenylsulphonyl chloride (pipsyl chloride). The picrates or pipsyl compounds are counted on standard plates at infinite thickness in a gas flow counter. Repeated recrystallizations and treatments with activated charcoal are then performed. When the radioactivity is stable through three recrystallizations purity is considered to have been achieved. The samples to be assayed for total imidazoleacetic acid are first hydrolysed in a sealed glass tube for 5 hr. at 160° in N hydrochloric acid with the carrier included. This hydrolysis removes the ribose that is conjugated to part of the imidazoleacetic acid.

The excretion of total [^{14}C] was measured as described by Schayer and Cooper (1956).

TABLE I

EXCRETION OF [^{14}C] IN URINE AFTER INJECTION OF [^{14}C]HISTAMINE

The [^{14}C] activity is expressed in terms of μg. [^{14}C]histamine.

Patient	A			B			C		
Injected [^{14}C] histamine (μg.)	11·5	10·2	11·5	11·5	11·5	11·5	11·5	11·5	11·5
Treatment	None	Iproniazid	Aminoguanidine	None	Iproniazid	Aminoguanidine	None	Iproniazid	Aminoguanidine
[^{14}C] activity in urine 0–12 hr. after injection	7·3	5·7	7·9	7·3	7·6	7·2	8·7	4·9	9·2
[^{14}C] activity in urine 12–24 hr. after injection	1·6	2·7	1·3	1·6	1·1	1·0	1·5	4·7	1·2
Total: [^{14}C] activity in urine 0–24 hr. after injection	8·9	8·4	9·2	8·9	8·7	8·2	10·2	9·6	10·4

RESULTS

Table I shows that by far the largest amount (71 to 90%) of the injected [^{14}C] was excreted during the first 24 hr. after the injection. The greater part of this [^{14}C] came out during the first 12 hr. However, in patients A and C, during treatment with iproniazid, the excretion of [^{14}C] seemed to be somewhat delayed. This may have been due to the fact that in these patients the urinary volumes were comparatively small during the first 12 hr. after the injection of [^{14}C]histamine during iproniazid treatment.

The analyses for radioactive metabolites of histamine in the two urine specimens are shown in Table II. Table III summarizes the main pathways for the catabolism of injected [^{14}C]-histamine, and the effect of aminoguanidine and iproniazid on them.

The Metabolism of [^{14}C]Histamine Without Treatment.—The pattern of urinary metabolites of [^{14}C]histamine in these three psychiatric patients was not significantly different from that in non-pregnant women (Nilsson *et al.*, 1959) or from that in normal men (Schayer and Cooper, 1956). The methylated compounds occurred in slightly higher amounts in the present study, but the difference is rather small. It is seen from the tables that imidazoleacetic acid and methyl-imidazoleacetic acid are the most important metabolites.

The Effect of Aminoguanidine.—It may be seen that aminoguanidine treatment blocked the transformation of histamine to imidazoleacetic acid. The inhibition was the same with 0.1 mg./kg. as with 1.0 mg./kg. Patient C, who was given a fourth injection of [^{14}C]histamine while receiv-

TABLE II

RADIOACTIVE METABOLITES OF [^{14}C]HISTAMINE IN URINE AS % OF EXCRETED [^{14}C]

The upper figures are those from the 0 to 12 hr. urine samples, the lower figures from the 12 to 24 hr. samples.

Patient	A			B			C		
Treatment	None	Iproniazid	Aminoguanidine	None	Iproniazid	Aminoguanidine	None	Iproniazid	Aminoguanidine
Histamine	1	5	3	2	4	0	2	4	0
	0	0	0	0	2	0	1	2	0
Imidazoleacetic acid (total)	26	3	2	26	5	2	23	4	2
	15	4	6	11	6	9	19	8	12
Methylhistamine	6	70	22	8	57	10	9	70	18
	0	41	5	1	34	4	1	46	3
Methylimidazoleacetic acid	60	13	71	68	30	61	60	22	77
	51	47	94	53	61	96	56	41	80
% of radioactivity recovered	93	91	98	104	96	73	94	100	97
	66	92	105	65	103	109	77	97	95

TABLE III

PATHWAYS IMPORTANT FOR [^{14}C]HISTAMINE CATABOLISM AND THE EFFECTS OF AMINOGUANIDINE AND IPRONIAZID

The figures show the range of values for the three patients studied, and refer to the whole 24-hr. period after injection of [^{14}C]histamine.

Enzyme	Metabolic Product Found in Urine	As % of Injected [^{14}C]		
		No Treatment	Iproniazid	Aminoguanidine
Histaminase (diamine oxidase)	Imidazoleacetic acid	17–20	2–5	2–3
Methylating enzyme	Methylhistamine	4–7	41–52	6–16
Methylating enzyme + oxidase	Methylimidazoleacetic acid	45–53	20–26	46–70

ing the same amounts of aminoguanidine by mouth as had been previously given subcutaneously, excreted [^{14}C]imidazoleacetic acid representing 2% and 0% respectively of the amount of radioactivity excreted during the two periods of urine collection. Thus, aminoguanidine sulphate seems to be effective when given by mouth.

The inhibitory effect of aminoguanidine indicates strongly that histaminase (diamine oxidase) is involved in the transformation of histamine to imidazoleacetic acid. It has been postulated from *in vitro* experiments that monoamine oxidase is important in this reaction (Zeller, Stern, and Blanksma, 1956), but monoamine oxidase is not inhibited by aminoguanidine.

The inhibition of histaminase by aminoguanidine does not lead to an increase in the urinary excretion of unchanged histamine. Instead more histamine is methylated as indicated by increased conversion to methylhistamine and methylimidazoleacetic acid.

The Effect of Iproniazid.—Treatment with iproniazid for 3 weeks causes conspicuous changes in histamine catabolism. The excretion of imidazoleacetic acid is reduced considerably, nearly as much as after treatment with aminoguanidine. The excretion of methylimidazoleacetic acid, not inhibited by aminoguanidine, is reduced by about 50% by iproniazid. The methylation of histamine increases just as much as it does after aminoguanidine, but since the oxidation to methylimidazoleacetic acid is partially blocked, methylhistamine is found as the most important urinary metabolite. These results indicate that iproniazid treatment inhibits both histaminase and the enzyme that oxidizes methylhistamine, while the methylating enzyme is not affected. Iproniazid has been found to be an efficient inhibitor of diamine oxidase *in vitro* (Lindahl, Lindell, Westling, and White, 1957; Blaschko, Friedman, Hawes, and Nilsson, 1959).

Discussion

The results are in full agreement with previous findings with the two enzyme inhibitors in animals (Schayer, 1959). We did not attempt to obtain a more complete inhibition by iproniazid with an increased dosage, since this drug has toxic effects. It appears that the inhibitor specificities of the enzymes concerned in histamine catabolism in man are not different from those in animals, which refutes the suggestion by Mitchell (1956).

As mentioned, the catabolism of [^{14}C]histamine in these psychiatric patients appeared to be normal. Some cases of chronic mental disease, notably schizophrenia, can tolerate large doses of histamine (Lucy, 1954), but in the patients studied here the response to histamine was entirely normal.

Iproniazid has profound effects on the catabolism of many amines in man. Thus Sjoerdsma, Gillespie, and Udenfriend (1959) found the conversion of 5-hydroxytryptamine, administered orally, to 5-hydroxyindoleacetic acid to be diminished by 33 to 55% after iproniazid, 50 mg. three times daily; this indicates that such a dose of iproniazid produces an inhibition of monoamine oxidase. Resnick (1959) concluded, from studies with radioactive adrenaline, that this dose level of iproniazid produces a 50% decrease of monoamine oxidase activity in man.

It appears therefore that iproniazid, 50 mg. three times daily, will reduce monoamine oxidase activity by about 50%. In the present study a 50% reduction of the oxidation of methylhistamine to methylimidazoleacetic acid was seen after iproniazid. This may be taken as indirect evidence that methylhistamine is oxidized by monoamine oxidase (Lindell and Westling, 1957). The lack of effect of aminoguanidine speaks against the possibility that histaminase (diamine oxidase) oxidizes methylhistamine in the body. *In vitro* methylhistamine is a good substrate of histaminase (Lindell and Westling, 1957).

Various investigators have tried to explain the central nervous effects of iproniazid as a result of an interference with the metabolism of 5-hydroxytryptamine and/or the catechol amines. It is therefore important to remember that iproniazid interferes with the catabolism of many other amines such as histamine.

To test the remote possibility that the therapeutic effect of iproniazid in patient C was connected with histaminase inhibition we gave aminoguanidine for 3 weeks, with no effect on the patient's mental depression. Later, iproniazid therapy was started again, with a good therapeutic result.

In order to evaluate the rate of formation and release of histamine in man *in vivo* it appears essential to develop methods for the determination of metabolic products of non-radioactive histamine. Imidazoleacetic acid is not a suitable metabolite for this purpose since the greater part of it is conjugated with ribose and hydrolysis of the riboside without carrier may cause losses in extraction. These losses are probably of no consequence with the method used in this paper since they should affect the carrier and the radioactive compound in equal proportions. Furthermore, imidazoleacetic acid may arise from

histidine by a pathway which does not involve histamine (Baldridge and Tourtellette, 1958; Lindell and Schayer, 1958b). Inferences about the histamine formation from analyses of urinary imidazoleacetic acid, free or conjugated, may therefore be entirely misleading. It would seem more promising to determine the methylated histamine derivatives. Of these, methylhistamine is probably more easily measurable. It is therefore of interest to know that after treatment with iproniazid a large percentage of injected histamine will be found in the urine as methylhistamine, and the determination of the urinary output of methylhistamine may be a measure of the total amount of histamine released or formed in such a patient.

We wish to thank Dr. Gayler White, Chief Physician at Lillhagen's Hospital, for his interest. Credit is due to the nurses of Ward 6B for ensuring adequate urine collection, and to Miss Maj-Britt Johansson for skilful technical assistance.

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SUPERSENSITIVITY OF THE SUBMAXILLARY GLAND FOLLOWING EXCLUSION OF THE POSTGANGLIONIC PARASYMPATHETIC NEURONE

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The repeated subcutaneous injection of four different parasympatholytic agents into cats has been found to cause a supersensitivity of the submaxillary glands to adrenaline. The fact that the level of sensitivity reached is the same with all four drugs is taken as evidence that the supersensitivity is due to the absence of an action of acetylcholine on the gland cells. This acetylcholine can only in part be that released by the secretory impulse, for the supersensitivity produced is more marked than that caused by preganglionic parasympathetic denervation. It is assumed that the postganglionic cholinergic neurone exerts some action of its own on the gland cell. Sensitization above the level reached after decentralization ensues when such an action is abolished by parasympatholytic agents. Degenerative section of the postganglionic fibres would also exclude this action, but for anatomical reasons this cannot be made complete. The supersensitivity which follows this operation is greater than after decentralization but less than that produced by treatment with atropine-like drugs.

The supersensitivity to various secretory agents acquired by denervated submaxillary gland cells can to a great extent be attributed to the fact that the cells have been deprived of the secretory impulses from the central nervous system, which normally impinge upon them. Evidence for this view can be found in the following observations: supersensitivity develops, both when the flow of secretory agents is reduced by cutting some of the afferent fibres of the secretory reflex arc, and when it is abolished by treatment of the experimental animal for some time with ganglionic blocking or parasympatholytic agents; furthermore, the supersensitivity caused by denervation disappears when the gland is supplied with injected secretory agents such as pilocarpine, carbachol, or adrenaline (Emmelin and Muren, 1950, 1951, 1952; Emmelin, 1952, 1956, 1959).

Those experiments in which parasympatholytic agents were used to create a supersensitivity deserve special attention. When such a drug, Hoechst 9980 ($\alpha\alpha$ - diphenyl - γ - piperidinobutyramide), was given subcutaneously for some weeks to cats, the supersensitivity of the submaxillary gland to adrenaline which ensued was found to exceed that caused by cutting the preganglionic parasympathetic fibres of the chorda tympani (Emmelin and Strömblad, 1957). The hypothesis was put forward that the drug not only deprives the gland cells of secretory impulses but in addition abolishes an action on the cells of acetylcholine continuously leaking from the endings of the postganglionic neurone (Emmelin and Strömblad, 1957, 1958).

In order to test this hypothesis two types of experiments, described in the present paper, were carried out.

The effect of postganglionic parasympathetic denervation on sensitivity was studied. The ideal experiment would be to compare the level of sensitivity produced by a parasympatholytic drug with that attained after complete section of the postganglionic parasympathetic fibres. Unfortunately this is not feasible, since many of the synapses are situated within the gland and the postganglionic fibres are therefore not anatomically accessible. An attempt could only be made to remove those nerve cells which are found close to the hilum of the gland and to compare the effect of this operation with that caused by section of the preganglionic fibres or treatment with atropine-like drugs.

In the second series of experiments a number of parasympatholytic agents were given to produce a supersensitivity. It was thought that if various different drugs of this pharmacological

group were found to create a supersensitivity surpassing that caused by section of the chorda this would support the view that the additional sensitization could be attributed to the abolition of the action of a cholinergic mechanism on the gland cells.

METHODS

Cats were used as experimental animals. The level of sensitivity of the submaxillary glands to secretory drugs was estimated in one and the same cat about once weekly over a period of several months; after initial ether the cats were anaesthetized with hexobarbitone, injected intracardially, and the two submaxillary ducts cannulated from the mouth (Emmelin and Muren, 1952). To elicit secretion, standard doses of adrenaline were injected intracardially. This was the only test drug used in cats treated with parasympatholytic agents. In the denervation experiments acetylcholine, methacholine, and synephrine were given as well. Care was taken not to inject acetylcholine and methacholine immediately after the administration of hexobarbitone because of the slight atropine-like effect of the latter drug (Emmelin, 1941).

Preganglionic denervation was made by cutting the chorda or chorda-lingual nerve. For partial postganglionic denervation the chorda fibres were traced along the salivary ducts, under the dissecting microscope, and cut as near the gland as possible. The distal end of the piece of nerve cut out was examined histologically for ganglion cells. This was kindly done by Dr. Dora Jacobsohn of Lund. The operation was done aseptically under hexobarbitone anaesthesia.

The following parasympatholytic drugs were used: lachesine chloride [(2-benziloyloxyethyl)ethyldimethylammonium chloride], Hoechst 9980, isopropamide iodide [Tyrimid; (3 - carbamoyl - 3,3 - diphenylpropyl)diisopropylmethylammonium iodide], and methscopolamine nitrate (hyoscine methonitrate). I am very grateful to Professor Burn, of Oxford, for a gift of lachesine. Hoechst 9980 was kindly supplied by A. B. Webass, Gothenburg, isopropamide by A. B. Leo, Hälsingborg, and methscopolamine by A. B. Pharmacia, Uppsala. The doses of the drugs used and further technical details are given below.

Some cats were finally studied in acute experiments under chloralose anaesthesia as described below.

RESULTS

Partial Postganglionic Denervation

After the dissection of the chorda towards the gland a supersensitivity developed which was more pronounced than that of a gland preganglionically denervated. This is illustrated in the experiment of Fig. 1, in which the chorda of the right submaxillary gland was dissected and the left chorda-lingual nerve cut at the same time. The supersensitivity resembled that seen after preganglionic section in the following respects: it developed with the same time course; the threshold dose was lowered, and the secretion time increased; it was unspecific, so that sensitization to adrenaline, synephrine, acetylcholine, and methacholine was seen. The difference was that the threshold was even lower than after preganglionic denervation, the secretion period for a certain dose longer, and the number of drops obtained after a moderate dose greater. With large doses the responses of the dissected and the contralateral, preganglionically denervated gland were about the same. In two cats the glands were cut out more than a month after the operations. They were found to weigh 0.705 (dissected) and 0.756 g. (chorda-lingual cut) in one animal, and 0.596 and 0.610 g. respectively in the other. The weight of a normal gland is about 1 g. (Emmelin, Jacobsohn, and Muren, 1951). Both glands had thus atrophied, and to about the same extent; this corresponds to the fact that the maximal secretory rate of the two glands was about the same.

In the dissection ganglion cells were removed. This was shown by the following observations:

(*a*) The distal part of the excised piece of nerve was often thicker than the rest of the chorda, and when squeezed in forceps gave the same feeling as, for instance, a superior cervical ganglion. Histological examination revealed the presence of numerous nerve cells.

(*b*) Eserine, injected through the duct towards the gland, had less secretory effect on the dissected

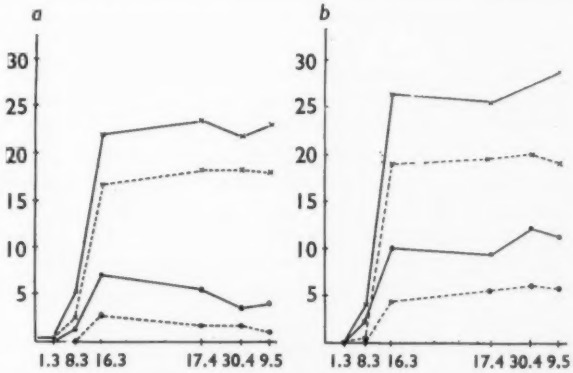

FIG. 1.—Supersensitivity following dissection and removal of the chorda towards the gland (solid lines) and simultaneous section of the contralateral chorda-lingual nerve (broken lines). Abscissa: dates (day and month) when the sensitivity was tested; ordinate: drops of saliva. *a*, Responses to 5 μg./kg. adrenaline (crosses) and 5 μg./kg. acetylcholine (rings); *b*, responses to 2 mg./kg. synephrine (crosses) and 2 μg./kg. methacholine (rings).

side than on the side of preganglionic section, in spite of the fact that the gland of the former side was more sensitive to acetylcholine than that of the other side. Eserine is assumed to act by preservation of acetylcholine leaking from the endings of the postganglionic neurone (Emmelin and Strömblad, 1958). Such an experiment is shown in Fig. 2. The smaller dose of eserine given

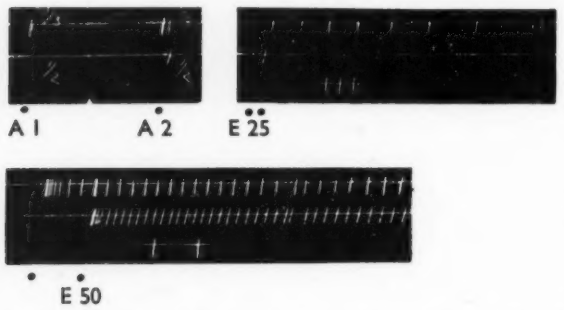

FIG. 2.—Chloralose anaesthesia. Secretory effects of acetylcholine (A 1=1 μg./kg., A 2=2 μg./kg.) and eserine (E 25=25 μg., E 50=50 μg.) 3 weeks after dissection of the chorda (upper line of the tracing) and section of the contralateral chorda-lingual nerve (lower line). Acetylcholine was given intravenously. Eserine, dissolved in 0.1 ml. saline solution, was injected during 5 sec. through a rubber connexion attached to the salivary cannula. The rubber tube was clamped distally during the injection and the following 5 sec. The dots mark the time of injection of the drugs. For eserine, the first dot corresponds to the injection into the duct of the dissected gland, the second into the other duct. Time in min. (in the lower tracing the drum was run at a higher speed).

in this experiment caused a secretion from the gland on the dissected side only (the single drop from the other side is probably part of the fluid injected which was expelled); this corresponds to the greater sensitivity of that side towards acetylcholine shown in the first part of the tracing. When the dose of eserine was raised, both glands secreted. The flow was now more rapid from the gland subjected to preganglionic denervation only. It could be objected that the higher dose of eserine could have a paralytic effect on the more sensitive gland; this was, however, not the case, for 100 and 200 μg. of eserine were found to cause a similar secretion to 50 μg. both on this gland and on the contralateral one. The conclusion from these experiments is that some postganglionic cholinergic fibres had degenerated on the dissected side.

(*c*) Nicotine, injected intravenously, was found to have less secretory effect on the dissected side than after mere preganglionic denervation. Dihydroergotamine was given in advance to abolish a secretory action of sympathin released by the nicotine from the adrenergic neurone or the adrenal medulla, and the experiments were carried out immediately after the operations in order to avoid the complication caused by sensitization.

That the postganglionic denervation was incomplete, as expected, was evident from the fact that eserine and nicotine still caused a marked secretion on the dissected side.

Postganglionic sympathetic fibres are known to enter the gland with the artery at the hilum. Section of these fibres is followed by a supersensitivity which can be superimposed upon that caused by cutting the chorda. Although care was taken not to touch the tissue surrounding the artery it seemed desirable to ascertain that the sensitization above the chorda level was not merely due to damage to sympathetic fibres. Two types of control experiments were carried out. (1) In some cats the sympathetic trunk was exposed in the neck but not cut. The trunk was stimulated electrically and the secretory response noted. The chorda was then immediately dissected towards the gland and stimulation of the sympathetic trunk repeated. The second secretory response was found to be as big as the first one. The wounds were then sutured. A supersensitivity was found to develop which surpassed that produced by section of the chorda-lingual nerve. This could not be due to injury of the preganglionic sympathetic fibres during the stimulation, for section of the sympathetic trunk does not sensitize the submaxillary gland cells (Emmelin and Engström, 1960). (2) In some cats the superior cervical ganglion was extirpated bilaterally when the level of supersensitivity had reached a plateau about three weeks after the usual parasympathetic operations. The sensitivity was found to increase on both sides, and the gland on the dissected side still showed a more pronounced supersensitivity than the gland deprived of the preganglionic parasympathetic (and postganglionic sympathetic) innervation.

Treatment with Parasympatholytic Agents

The chorda on one side was cut, and when the supersensitivity had reached a maximum, treatment with the parasympatholytic drugs started. The drugs were given subcutaneously. Hoechst 9980 was injected once a day in a dose of 1 mg./kg. It was found to cause a higher supersensitivity to adrenaline than that produced by preganglionic parasympathetic denervation, as

described earlier (Emmelin and Strömblad, 1957). A similar result was obtained with isopropamide iodide, 1 mg./kg. a day. When the same dose of lachesine chloride was administered, the sensitivity of both glands started to rise, but after two weeks it was found to be lower than after one week. Obviously a tolerance to the drug had developed. The dose had to be raised successively in order to prevent a fall in sensitivity, and doses as high as 10 mg./kg., given twice a day, were eventually used in some cats. Similar results were obtained when atropine was used (Emmelin and Muren, 1950a, 1951). The experiment shown in Fig. 3 demonstrates the decline of the sensitivity

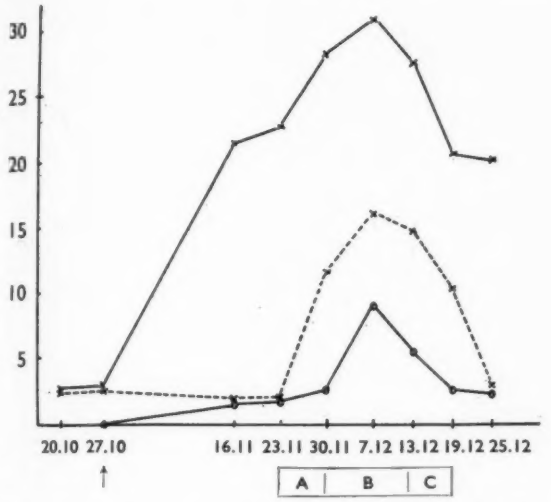

FIG. 3.—Abscissa; date (day and month) of estimation of the sensitivity, ordinate: drops of saliva secreted in response to 5 μg./kg. (crosses) and 1 μg./kg. (rings) of adrenaline for the right gland (solid lines) and for the left gland (broken line; no response to 1 μg./kg.). At the arrow the right chorda tympani was cut. Treatment with lachesine chloride, A: 1 mg./kg. once a day, B: 1 mg./kg. twice a day, C: 2 mg./kg. twice a day.

curves in spite of a moderate increase in the dose of lachesine. Nevertheless, it is apparent from the Fig. that a higher supersensitivity can be reached by treatment with this drug than that caused by preganglionic denervation. Methscopolamine nitrate was initially given daily in doses of 1 mg./kg. Since there seemed to be a slight tendency for a tolerance to develop, it was often found suitable to raise the dose to 2 mg./kg. a day after 1 or 2 weeks. A supersensitivity above that caused by section of the chorda was found to ensue.

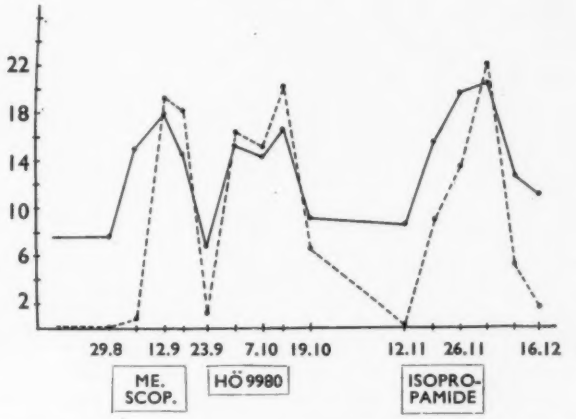

FIG. 4.—Secretory responses from the right (solid line) and the left gland (broken line) to a standard dose of 10 μg./kg. adrenaline. The right chorda had been cut 3 weeks before the experiment started. The dose of the three drugs was 1 mg./kg. a day.

In some cats it was possible to try several parasympatholytic drugs in the same animal. These experiments allow the conclusion to be drawn that all the drugs produce about the same high level of supersensitivity. Such an experiment is shown in Fig. 4, which demonstrates that methscopolamine, Hoechst 9980 and isopropamide sensitize both glands far above the level caused by the section of the chorda and that all three drugs create about the same degree of supersensitivity. There might be a slight difference

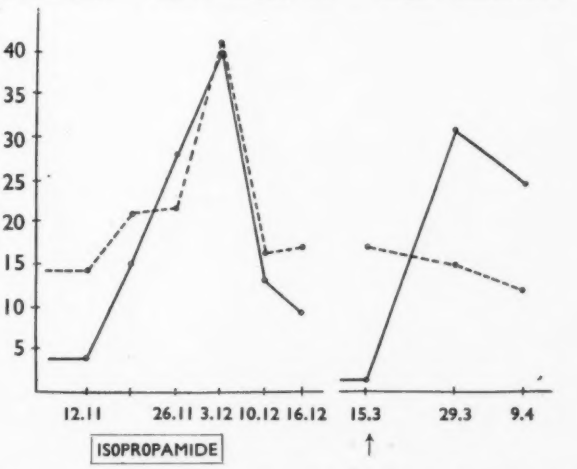

FIG. 5.—Secretion from the right (solid line) and left gland (broken line) caused by 5 μg./kg. adrenaline. The left chorda had been cut 3 weeks before the experiment started. Isopropamide iodide was given in a dose of 1 mg./kg. once a day. At the arrow the right chorda was dissected towards the gland.

between the responses of the right and left gland treated with blocking agents in this cat; this could be due to the fact that section of the chorda causes some atrophy of the gland, an effect which treatment with parasympatholytic agents does not seem to give (Emmelin, Jacobsohn, and Muren, 1951).

Comparisons were made between the supersensitivity caused by treatment with parasympatholytic agents and that produced by dissecting the chorda towards the gland. Fig. 5 shows that the operation produced a supersensitivity which was more pronounced than that following section of the chorda. It was, on the other hand, less marked than that caused by treatment with the parasympatholytic agent, which was to be expected since the postganglionic denervation was incomplete.

Discussion

The finding that four different parasympatholytic agents sensitize the submaxillary gland to the same level suggests that they act by virtue of their parasympatholytic properties only. Since the level of supersensitivity reached by far exceeds that attained by disconnecting the gland from the central nervous system via the chorda tympani it must be assumed that the drugs deprive the gland not only of acetylcholine released by the secretory impulse but in addition of some other influence exerted by a cholinergic mechanism. It is reasonable to focus interest on the postganglionic parasympathetic neurone. This neurone must then be assumed to have some action on the glandular cell apart from that due to the secretory impulse from the central nervous system, an action which persists after section of the preganglionic fibres. This postulated action of the neurone on the gland cell must be mediated by acetylcholine released in subthreshold concentration as regards secretion, since the gland does not secrete under ordinary conditions after section of the chorda. The fact that small amounts of acetylcholine can be detected in a perfusate of an eserinized gland even when the chorda is not stimulated (Emmelin and Muren, 1950b) is of interest in this connexion. Recent experiments with eserine (Emmelin and Strömblad, 1958) support the view that there is a continuous leakage of acetylcholine from the endings of the postganglionic neurone. The sensitization above the chorda level caused by parasympatholytic agents suggests that the leaking acetylcholine, although insufficient to cause secretion, exerts some action on the gland cell, the removal of which manifests itself in supersensitivity. The gland can be deprived of this action by treatment with atropine-like drugs. Another way of removing this influence would be to cause the postganglionic fibres to degenerate. Unfortunately this can only be done with some fibres; correspondingly, the sensitivity is then only raised to a level below that reached after treatment with parasympatholytic drugs.

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Brit. J. Pharmacol. (1960), **15**, 361.

BRITISH PHARMACOLOGICAL SOCIETY AND SCANDINAVIAN PHARMACOLOGICAL SOCIETY

COPENHAGEN MEETING, 26th, 27th, and 28th JULY, 1960

SYMPOSIUM ON "THE PHARMACOLOGY OF MEMBRANES"

Dr. POUL KRUHØFFER, Institute of Physiology, University of Copenhagen: "Physiology of Membranes."

Dr. KARL ZERAHN, Institute of Biological Chemistry, University of Copenhagen: "The Sodium Pump."

Dr. JENS CHR. SKOU, Institute of Physiology, University of Aarhus: "The Effect of Drugs on the Cell Membrane."

Dr. STEPHEN THESLEFF, Institute of Pharmacology, University of Lund: "The Influence of Innervation of Membrane Properties."

DEMONSTRATIONS

1. **P. Lund Nilsen** (*Pharmacological Laboratory, Meco-Dumex, 37 Prags Boulevard, Copenhagen, S*).

Determination of local anaesthetics by the mouse-tail method.

COMMUNICATIONS

1. **A. L. A. Boura** and **A. F. Green** (*The Wellcome Research Laboratories, Beckenham, Kent*).

New adrenergic neurone blocking agents.

2. **A. L. A. Boura, A. McCoubrey** and **W. G. Duncombe** (*The Wellcome Research Laboratories, Beckenham, Kent*).

The distribution of bretylium and other quaternary ammonium salts in the peripheral sympathetic nervous system.

3. **A. K. Armitage, Janet Boswood** and **B. J. Large** (*May & Baker, Dagenham, Essex*).

Some pharmacological properties of a new xanthine derivative with potent bronchodilator and coronary dilator properties.

4. **A. Herxheimer** (*Department of Pharmacology, The London Hospital Medical College, London, E.*1).

Some interactions of morphine and nalorphine in mice.

5. **D. J. Jenden** (*introduced by* **W. L. M. Perry**) (*Department of Pharmacology, University of California Medical Center, Los Angeles* 24, *California*).

The effect of ryanodine on relaxing systems in skeletal muscle.

6. **E. M. Vaughan Williams** (*Department of Pharmacology, South Parks Road, Oxford*).

A comparison of the effects of 5-hydroxytryptamine, histamine and anaphylaxis on cardiac intracellular potentials and contractions.

7. **C. Rerup** (*Department of Pharmacology, University of Lund, Sweden*).

Difference parameters in the twin cross-over test (insulin and corticotrophin) and their consequences for assay validity.

8. **K. Martindale, G. F. Somers** and **C. W. M. Wilson** (*University of Liverpool and the Distillers Co.* (*Biochemicals*) *Liverpool*).

Some factors affecting the production of gastric ulcers in guinea-pigs and rats.

9. **M. W. Parkes** and **Joan T. Pickens** (*Pharmacological Laboratory, Research Department, Roche Products, Welwyn Garden City, Herts.*).

Conditions governing the assay of analgesics by the writhing response of mice to phenylbenzoquinone.

10. **A. W. Lessin** (*introduced by* **M. W. Parkes**) (*Pharmacological Laboratory, Research Department, Roche Products, Welwyn Garden City, Herts.*).

Species differences in the effect of barbiturate habituation on sensitivity to drugs.

11. **H. M. Cabot, R. Caldeyro-Barcia, R. J. Fitzpatrick** and **C. Mendez-Bauer** (*Servicio Fisiologica Obstetrica of the University of Uruguay*).

The estimation of the concentration of oxytocin in blood.

12. **D. G. Hardy, R. E. Lister** and **E. S. Stern** (*Research Department, J. F. Macfarlan & Co., Edinburgh*).

Structure activity relationships among some new analogues of pethidine.

13. **F. Hobbiger** (*Department of Pharmacology, Middlesex Hospital Medical School, London, W.*1).

Protection by oximes against poisoning by organophosphates which form dimethylphosphoryl acetylcholinesterase.

14. **E. E. Daniel** (*introduced by* **M. Weatherall**) (*Department of Pharmacology, The London Hospital Medical College, London, E.*1).

Potassium fluxes in the rat uterus *in vitro* during metabolic inhibition.

15. **D. Elmqvist** and **T. R. Johns** (*introduced by* **S. Thesleff**) (*Department of Pharmacology, University of Lund, Sweden*).

Electrophysiological properties of human skeletal muscle.

16. **Eva M. Kovacs** and **G. A. H. Buttle** (*Department of Pharmacology, The School of Pharmacy*, 29-39 *Brunswick Square, London, W.C.*1).

Immunological aspects of transplanted tumours.

17. **L. C. Blaber** and **W. C. Bowman** (*Department of Pharmacology, The School of Pharmacy*, 29-39 *Brunswick Square, London, W.C.*1).

Some effects of anticholinesterases in cat and hen muscle.

18. **P. S. J. Spencer** (*introduced by* **G. B. West**) (*Department of Pharmacology, The School of Pharmacy*, 29-39 *Brunswick Square, London, W.C.*1).

Thyroid hormone and hypersensitivity.

19. **A. W. Cuthbert** (*introduced by* **G. B. West**) (*Department of Pharmacology, The School of Pharmacy*, 29-39 *Brunswick Square, London, W.C.*1).

Some studies on chick amnion.

20. **B. P. Block** (*introduced by* **G. A. H. Buttle**) (*Department of Pharmacology, The School of Pharmacy*, 29-39 *Brunswick Square, London, W.C.*1).

Factors affecting carbohydrate metabolism in mice.

21. **Shirley A. P. Price** and **G. B. West** (*Department of Pharmacology, The School of Pharmacy*, 29-39 *Brunswick Square, London, W.C.*1).

An unidentified pharmacologically active substance from cerebral hemispheres.

22. **D. F. Elliott, E. W. Horton** and **G. P. Lewis** (*National Institute for Medical Research, Mill Hill, London, N.W.*7).

Problems arising from the structure of bradykinin.

23. **G. P. Lewis** (*National Institute for Medical Research, Mill Hill, London, N.W.*7).

The possible role of bradykinin in the inflammatory response.

24. **F. M. Sullivan** (*Department of Pharmacology, Guy's Hospital Medical School, London, S.E.*1).

The effect of cortisone on the antituberculous activity of isoniazid.

25. **P. D. Mulcahy** (*introduced by* **J. P. Quilliam**) (*Department of Pharmacology, The Medical College of St. Bartholomew's Hospital, London, E.C.*1).

The effects of thioridazine in man.

26. **B. W. Payton** (*introduced by* **J. P. Quilliam**) (*Department of Pharmacology, The Medical College of St. Bartholomew's Hospital, London, E.C.*1).

The effect of some central nervous system depressants and hexamethonium on neuromuscular transmission.

27. **D. F. J. Mason** (*Department of Pharmacology, The Medical College of St. Bartholomew's Hospital, London, E.C.*1).

The stimulant action of certain anticholinesterases on sympathetic ganglia.

28. **Sybil Lloyd** and **Mary Pickford** (*Physiology Department, University of Edinburgh*).

Effect of autonomic nervous blockade on the action of oxytocin on the blood-pressure of the hen.

29. **D. H. Sproull** (*National Institute for Medical Research, Mill Hill, London, N.W.*7).

Effects of some hydroxybenzoates on rat liver glutathione concentration.

30. **A. Wretlind** and **P. Westerholm** (*Department of Pharmacology, Karolinska Institutet, Stockholm* 60).

Emulsions of synthetic triglycerides: a new group of agents with circulatory and respiratory effects.

31. **W. D. Alexander, Sheenah K. Bisset** and **J. Crooks** (*introduced by* **A. G. Macgregor**) (*Department of Biochemistry and Gardiner Institute of Medicine, Western Infirmary, Glasgow*).

A method for comparing the calorigenic effect and duration of the " latent period " of the thyroxine analogues using human leucocytes.

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The influence of some psychotropic drugs on the social behaviour of male sticklebacks.

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The potentiation of analgesics by some tranquillizers and sedatives.

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Aspects of bemegride action.

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The action of bretylium on the peristaltic reflex in the isolated guinea-pig ileum.

36. **Mary F. Lockett** and **K. E. Eakins** (*Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.3*).

Procedures causing the appearance of traces of an isoprenaline-like compound in plasma.

37. **M. K. Paasonen** (*Department of Pharmacology, University of Helsinki*).

Inactivation of 5-hydroxytryptamine released from blood platelets *in vitro*.

38. **M. M. Airaksinen** (*Department of Pharmacology, University of Helsinki*).

Aspects in the metabolism and excretion of 5-hydroxytryptamine.

39. **Anja Tissari** (*Department of Pharmacology, University of Helsinki*).

5-Hydroxytryptamine in the foetal and young guinea-pig.

40. **R. S. Stacey** (*Department of Pharmacology and Therapeutics, St. Thomas's Hospital Medical School, London, S.E.1*).

The uptake of 5-hydroxytryptamine by blood platelets.

41. **J. G. A. Pedersen** (*Aktieselskabet Pharmacia, 48 Lindealle, Copenhagen*).

Pharmacology and preliminary clinical trial of N-(γ-trimethyl-ammonium-n-propyl)-N-methyl-3-chloro-camphidinium sulphate, a ganglionic blocking agent.

42. **J. Fakstorp** (*Aktieselskabet Pharmacia, 48 Lindealle, Copenhagen*).

Structure activity relations in three analogous series of amines with ganglion blocking action: terpenes, cyclohexanes and propanes.

43. **R. W. Foster** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, King's College, London, W.C.2*).

Cocaine, guanethidine, sympathomimetic amines, and the adrenergic nerve endings of the isolated guinea-pig trachealis.

44. **G. Paterson** (*introduced by* **G. Brownlee**) (*Department of Pharmacology, King's College, London, W.C.2*).

The effect of caffeine and depolarizing drugs on the membrane potential and the contracture induced in mammalian muscle.

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The effect of central nervous stimulants and depressants on the release of 5-hydroxytryptamine from the isolated gut.

46. **B. Ablad** and **G. Johnsson** (*Farmakologiska Institutionen, Göteborgs Universitet, Göteborg C*).

Comparison of the effects of hydrallazine and sodium nitrite on the resistance and capacity vessels of the human forearm.

47. **Aimo Pekkarinen** and **Eero Tala** (*Department of Pharmacology, Turku University, Turku, Finland*).

The controlling effect of drugs on the content of free 17-OHCS in the plasma of guinea-pigs and rats.

48. **Niilo Niemelä, Aimo Pekkarinen** and **Eero Sotaniemi** (*Department of Pharmacology, Turku University, Turku, Finland*).

The effect of psychic stress and psychopharmaca on the urinary excretion of the total 17-OHCS in guinea-pigs.

49. **R. Schneider, Hilary Bishop, G. Robinson** and **A. C. Frazer** (*Department of Medical Biochemistry and Pharmacology, The Medical School, Birmingham* 15).

Recent observations on the mechanism of the inhibitory effect of gluten on isolated intestine, and the nature of the inhibitory agent.

50. **V. Rønnov-Jessen** (*Kobenhavns Amts Sygehus, Hellerup*).

Blood volume and development of tolerance to ganglion blocking agents in treatment of hypertension.

51. **P. F. D'Arcy** and **J. J. Grimshaw** (*Research Division, Allen & Hanburys, Ware, Hertfordshire*).

The abnormal effects of low concentrations of some drugs on ciliary movement.

52. **P. F. D'Arcy, J. W. Fairbairn** and **J. J. Grimshaw** (*Research Division, Allen & Hanburys, Ware, Hertfordshire*).

The assessment of purgative activity using mice.

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Application of the pituitary push and pull principle of hypothalamus depressants.

54. **I. Møller Nielsen** and **I. Huus** (*Department of Pharmacology, Lundbeck & Co. A/S, Copenhagen*).

Monoamino-oxidase inhibition of iproniazid and β-p-chlorphenyl-mercapto-ethylhydrazine in liver and brain.

Brit. J. Pharmacol. (1960), **15**, 365.

THE RESPONSES OF THE *VENUS* HEART TO CATECHOL AMINES AND HIGH CONCENTRATIONS OF 5-HYDROXYTRYPTAMINE

BY

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(RECEIVED JANUARY 28, 1960)

The catechol amines excite the isolated heart of *Venus mercenaria* in a characteristic manner. This response was not obtained with phenethylamine, tyramine, ephedrine, or mescaline, nor with histamine, nor with the basic n-alkylamines. 5-Hydroxytryptamine had a distinctive effect at high concentrations (above 3×10^{-6} M) different from that at lower doses. The response to high concentrations was dominated by an increase in muscle tone. Hearts exposed to high concentrations of 5-hydroxytryptamine and other tryptamine analogues for long periods became tachyphylactic to low doses of these substances. However, high doses of 5-hydroxytryptamine (about 2×10^{-5} M) still excited the tachyphylactic heart, but the response was then like that to the catechol amines. When high bath temperatures rendered the heart insensitive to 5-hydroxytryptamine, high concentrations of this compound again had the catechol amine effect. The possibility of a physiological role for the catechol amines or high 5-hydroxytryptamine concentrations is discussed.

While studying structure-activity relations on the heart of *Venus mercenaria* (Greenberg, 1960), attention was drawn to the effects of some catechol amines and high concentrations of 5-hydroxytryptamine.

The responses of lamellibranch hearts to adrenaline and noradrenaline are varied (Krijgsman and Divaris, 1955; Welsh, 1953; Fänge, 1955; Gaddum and Paasonen, 1955). For example, most hearts are excited by adrenaline ; when treated with high concentrations they are arrested in systole. On the other hand, the oyster (Jullien, 1936) and *Anodonta cygnea* (Fänge, 1955) show negative inotropic effects when treated with adrenaline. Again, *Amblema peruviana* (Motley, 1934) and *Cardium edule* (Gaddum and Paasonen (1955) are arrested in diastole by high concentrations of catechol amines.

Only a few workers have tested both catechol amines and 5-hydroxytryptamine on the lamellibranch heart (Welsh, 1953 ; Fänge, 1955 ; Gaddum and Paasonen, 1955). In the species tested, the hearts have always been found to be more sensitive to 5-hydroxytryptamine than to adrenaline or noradrenaline. In only three species was there a notable qualitative difference between the two excitatory actions.

High concentrations of 5-hydroxytryptamine have never been studied, probably for the good reason that the physiological role of this substance as excitatory neurohumour (Welsh, 1957) is likely to be associated with near-threshold concentrations.

Tachyphylaxis to 5-hydroxytryptamine was demonstrated by Gaddum on guinea-pig ileum in 1953. It has since been observed in a number of mammalian preparations (Gaddum and Hameed, 1954), but has never been produced in molluscan preparations.

In the present study on the *Venus* heart the mode of excitation by catechol amines is examined over the entire range of action. High concentrations of 5-hydroxytryptamine are also tested and it is found that they can induce tachyphylaxis to moderate concentrations of this substance. When such desensitization has been established, however, high 5-hydroxytryptamine doses evoke responses similar to those of the catechol amines. The structure-activity relations of this response are briefly studied. The possibility that there is a physiological role for the catechol amines in *Venus* is suggested.

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METHODS

Preparation.—Large specimens of *Venus mercenaria* (clams; quahogs), obtained fresh from Narragansett Bay, were employed. The hearts were removed in the manner described by Welsh and Taub (1948). The excised ventricles were immersed in a jacketed, aerated 10 ml. bath and stretched between a stainless steel hook and an isotonic lever balanced either at 500 or 1,000 mg. The perfusion fluid in the bath proper was filtered sea water obtained from Woods Hole, Mass. Water from a reservoir, thermostatically maintained at 15°, was circulated through the bath jackets. Responses were recorded on a kymograph with a smoked drum.

Drugs were made up in standard solutions of 10^{-1} M to 10^{-3} M. These were serially diluted with distilled or sea water. The dose in a volume of 0.1 to 1.0 ml. was added directly to the bath fluid with a hypodermic syringe. Proper mixing was provided by the stream of aerating bubbles. The addition of 1.0 ml. of distilled water alone has no effect on the clam heart. Most doses are expressed as moles per litre, but concentrations of Brom LSD and Mytolon in the bath are given as g./ml.

The length of exposure of a heart to a particular dose varied between a min. and over an hr. depending upon the compound and the concentration used. At least 5 min. were always allowed between washing and the addition of the succeeding dose.

Benzoquinonium chloride (Mytolon; 2:5-bis (3′ diethylaminopropylamino)-benzoquinone bis benzyl chloride) (10^{-5} g./ml.) was maintained in the bath except during the washing process. This drug is an effective antagonist of acetylcholine in the *Venus* heart (Luduena and Brown, 1952). In these experiments benzoquinonium was used to improve the regularity of the beat by preventing the depression of the heart probably caused by endogenous acetylcholine. Benzoquinonium has no effect on the response of the heart to 5-hydroxytryptamine.

The Effect of High Bath Temperature on the 5-Hydroxytryptamine Effect.—By increasing the temperature of the water in the reservoir, that of the circulating water in the jackets could be raised to 30° or 35°. Temperature equilibrium between the jacket water and the sea water in the bath occurred in about 5 min. Ten min. were always allowed.

The Effect of pH Change on the Performance of the Isolated Venus Heart Preparation.—Aliquots of sea water were adjusted with hydrochloric acid or sodium hydroxide so that the *p*H varied from 4.2 to 9.0. In testing the effects of *p*H on the heart, normal sea water in the bath was rapidly withdrawn and replaced by the altered sea water. The hearts were equilibrated at the new hydrogen ion concentration for 15 min.

Specificity.—Brom LSD (2-brom-(+)-lysergic acid diethylamide) (10^{-5} g./ml.) is an antagonist of 5-hydroxytryptamine on the *Venus* heart (Welsh and McCoy, 1957). This antagonism is believed to be specific. In the present study Brom LSD is used to test the specificity, relative to 5-hydroxytryptamine, of the various exciter agents employed.

Drugs Used.—The following compounds were used in the course of this study: Tryptamine hydrochloride, 3,4-dihydroxyphenylethylamine (dopamine) hydrochloride (Mann Research Laboratories); 5-hydroxytryptamine creatinine sulphate (Nutritional Biochemicals); 2-brom-(+)-lysergic acid diethylamide (Sandoz, Inc.); phenethylamine, heptylamine, *n*-hexylamine, *n*-amylamine, *n*-butylamine, ethylamine (Eastman Kodak); tyramine (Abbott Laboratories); (−)-epinephrine bitartrate, levarterenol bitartrate monohydrate, benzoquinonium chloride (Sterling-Winthrop); ephedrine hydrochloride, mescaline hydrochloride (Hoffman-LaRoche); histamine diphosphate (General Biochemicals); bufotenine, 5-hydroxy-α-methyltryptamine, N′N′-dimethyltryptamine (Upjohn).

RESULTS

The Catechol Amine Effect.—The effects of adrenaline, noradrenaline and dopamine on the *Venus* heart were similar, but differed from those of 5-hydroxytryptamine.

The catechol amines produced a series of responses which varied qualitatively with concentration. There were two sorts of effects in the range of action. At the low-concentration end there occurred a decrease in amplitude with no chronotropic effect. Since these experiments were done in the presence of benzoquinonium this response could not have been due to a stimulation of the acetylcholine receptors of the heart. At higher concentrations an increase in tone, concomitant with an increase in frequency, predominated. At the extreme high end of the concentration range, the response was immediate systolic arrest.

The effects of adrenaline and noradrenaline were almost identical (Fig. 1). The pure negative inotropic effect was a relatively small part of the response occurring just at threshold doses (10^{-5} to 5×10^{-5} M) (Fig. 1a and c). A slight increase of the threshold dose by $1–2\times10^{-5}$ M resulted in a response which was a mixture of the negative inotropic and positive chronotropic effects as well as an increase in tone ($2–7\times10^{-5}$ M). A dose of adrenaline or noradrenaline 5 to 10 times larger than that which just increases the tone caused systolic arrest ($7–12\times10^{-5}$ M) (Fig. 1a).

The action of dopamine differed from that of adrenaline and noradrenaline in two respects. Firstly, the potency of dopamine, with regard to the increase of tone and frequency, was about 10 times that of adrenaline or noradrenaline (Fig. 1c). Secondly, the decrease in amplitude played a more prominent part in the response to dopamine. There was a good deal of variation, but a negative inotropic effect was always obtainable at 2 to 3×10^{-6} M (Fig. 1c). A decrease in amplitude of more than 50% was not unusual. Increases of tone

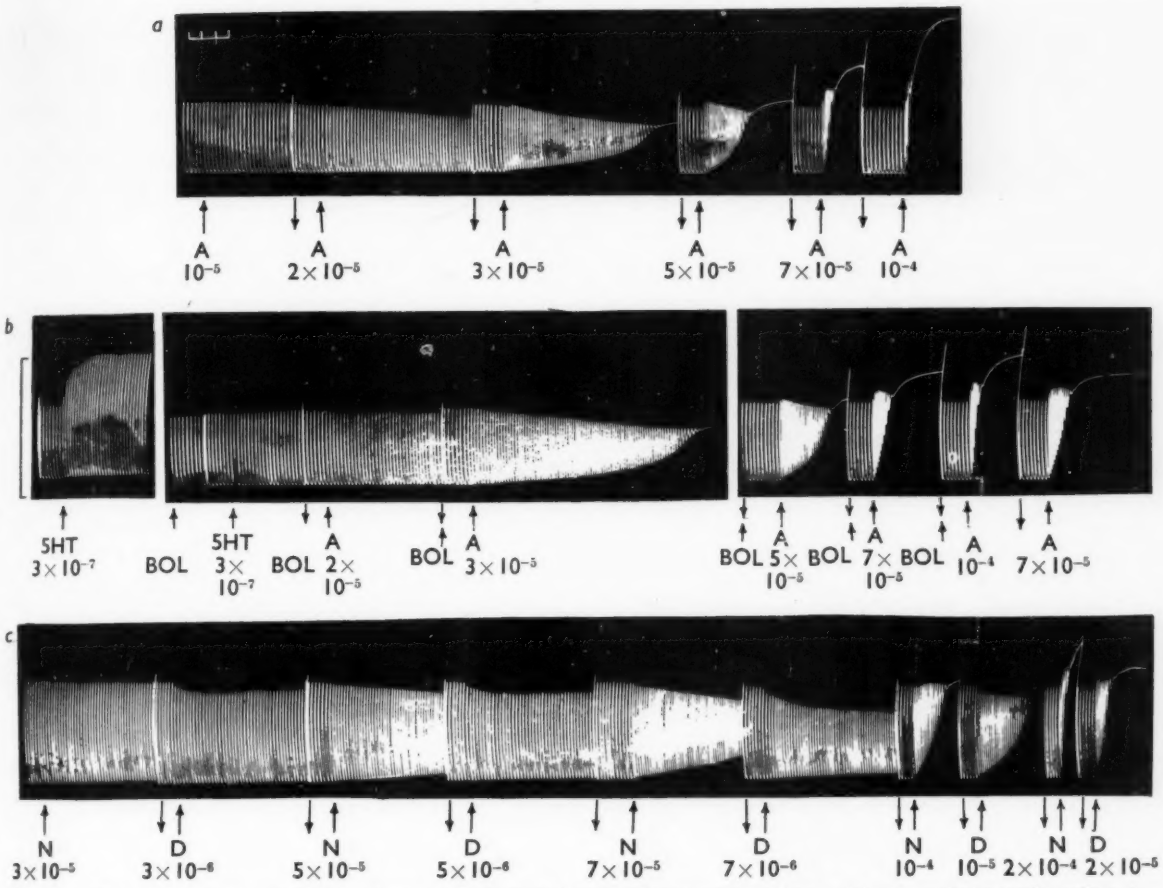


FIG. 1.—The response to catechol amines of the isolated *Venus* heart preparation. (*a*) The effect of adrenaline A. (*b*) Same preparation. The effect of adrenaline (A) in the presence of 2-bromo-(+)-lysergic acid diethylamide (BOL) (10^{-5} g./ml.). First dose of BOL added 20 min. before excitator agent (5-hydroxytryptamine (5HT)); maintenance doses immediately after washing and 5 min. before each dose of exciter. (*c*) Comparison of noradrenaline (N) and dopamine (D) on the same preparation. Benzoquinonium chloride (10^{-5} g./ml.) added to bath 5 min. before each dose. Downward-pointing arrows indicate washing. Dose: moles/litre. Tension: 1,000 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 5 cm.

were usually produced by a dose of 5 to 20 times greater than threshold.

The responses to catechol amines were not blocked by Brom LSD (10^{-5} g./ml.) (Fig. 1b); this distinguishes these responses from those to 5-hydroxytryptamine.

It is important to know whether the catechol amine effect is merely an unspecific response of the heart to high concentrations of drug or whether it results from an action at specific sites in the tissue. A partial answer to this question can be obtained by examining the responses of the heart to various analogues of the catechol amines.

Responses to Other Phenethylamine Analogues.—Tyramine and phenethylamine were tested at concentrations between 10^{-5} M and 10^{-4} M. The response of the heart was 5-hydroxytryptamine-like, although 500 to 4,000 times weaker. The positive inotropic effect was antagonized by Brom LSD. This surprising result is dealt with elsewhere (Greenberg, 1960).

The actions of ephedrine and mescaline were relatively feeble even at such high concentrations as 10^{-4} M to 10^{-3} M. The effects of these compounds were qualitatively similar to those of tyramine and phenethylamine. Mescaline had about one third, ephedrine one fiftieth the potency of phenethylamine. No attempt was made to block the actions of these two compounds with Brom LSD.

None of the above analogues of phenethylamine tested produced an effect resembling that of the catechol amines at any concentration.

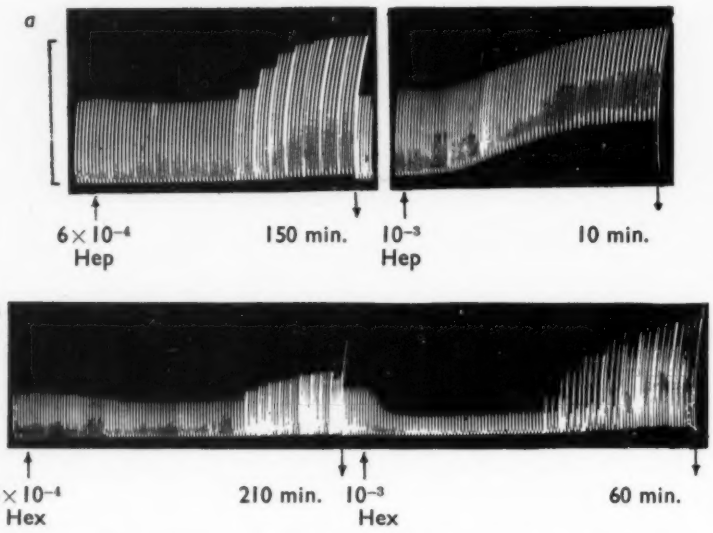


FIG. 2.—The response of the isolated *Venus* heart preparation to *n*-alkylamines. (*a*) Heptylamine (Hep). (*b*) Hexylamine (Hex). Total time for each response is indicated in min. Downward-pointing arrows indicate washing. Benzoquinonium chloride (10^{-5} g./ml.) was added to the sea water 5 min. before each dose. Dose: moles/litre. Tension: 500 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 5 cm.

Responses to Histamine (*4-Ethylamino Imidazole*).—Histamine is a well-known smooth muscle exciter. It acts occasionally on lamellibranch hearts in relatively high concentrations (Pilgrim, 1954 ; Gaddum and Paasonen, 1955). Histamine has a curious effect on the *Venus* heart. Threshold concentration, when there was any action at all, was about 10^{-6} M. The response was a rapid increase in amplitude of beat which fell off again fairly rapidly. The increase in amplitude was equivalent to that produced by about 4×10^{-9} M 5-hydroxytryptamine. Increasing doses of histamine, up to 10^{-4} M, did not increase the effect ; in fact, the response sometimes became smaller.

The action of histamine was not blocked by Brom LSD and hence histamine was not acting on the 5-hydroxytryptamine site. No sign of a catechol-amine-like effect has been observed in response to histamine.

Responses to n-*Alkylamines*.—The *n*-alkylamines tested excited the *Venus* heart in high concentrations. However, the mode of excitation was unique : neither like that of the catechol amines, nor hist-amine, nor 5-hydroxytryptamine.

Threshold, for amyl-, hexyl-, and heptylamine, was between 10^{-4} and 3×10^{-4} M. The effect was a transient decrease in amplitude lasting about 10 min. and followed by an increase in amplitude which took about an hour to develop (Fig. 2). Both the positive and negative inotropic effects increased only slightly with increasing doses up to 8×10^{-4} M. At 10^{-3} M the amines had a sudden violent effect the nature of which depended upon the compound used. With heptylamine there was a great increase of tone and a slowing of rate (Fig. 2). Hexylamine and amylamine produced an augmentation of the transient decrease and then a great irregularity of beat (Fig. 2). As might be expected the alkylamine effect was not blocked by Brom LSD (10^{-5} g./ml.).

The *n*-alkylamines with a chain length of less than five carbons had a smaller potency. Ethylamine and butylamine had about one third of the activity of hexylamine ; ammonia had one sixth to one tenth of the activity of hexylamine.

The perfusion fluid, sea water, is buffered naturally. In the course of storage, prior to use, the *p*H dropped to about 7.8. The addition to the bath of relatively large concentrations of these basic amines caused the *p*H to increase to as much as 8.4. Four heart preparations were tested to determine their tolerance to changes of hydrogen ion concentration. The normal functioning of the hearts was independent of *p*H. The frequency was stable at about 10 beats per min. between *p*H 5.9 and 8.6. The amplitude, while stable between *p*H 4.8 and 9.0, dropped noticeably at 4.2. Thus, between *p*H 7.8 and 8.4, the range employed in the study of the *n*-alkylamines, there was no *p*H-induced variation in the normal amplitude and frequency of the hearts.

The Effect of 5-Hydroxytryptamine.—The response of the *Venus* heart to 5-hydroxytryptamine has three components : an increase in amplitude, an increase in frequency, and an increase in the resting tone of the muscle. The relative importance of these components varies with the concentration (see Figs. 3 and 4).

Between threshold concentration (about 10^{-9} M) and moderately high doses (about 10^{-6} M) the response of the *Venus* heart to 5-hydroxytryptamine was mainly an increase in the amplitude of beat. There was also an increase in tone, which increases with concentration, and an increase in frequency which, however, was not dependable. A plot of the final amplitude of the response (measured from the original baseline) against log concentration is a

ERRATUM

In the paper by M. J. Greenberg in the September issue, p. 369, Fig. 3, the concentrations of 5-hydroxytryptamine should read from left to right 3×10^{-9} 3×10^{-8} 3×10^{-7} 3×10^{-6} 3×10^{-5}.

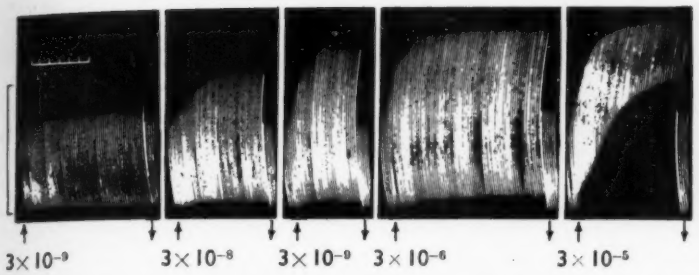


FIG. 3.—The effect of 5-hydroxytryptamine (5HT) on the isolated *Venus* heart preparation. Downward-pointing arrows indicate washing. Benzoquinonium chloride (10^{-5} g./ml.) added to sea water 5 min. before each dose of 5HT. Dose: moles/litre. Tension: 1,000 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 5 cm.

sigmoid curve which levels out at about 10^{-6} M and then rapidly increases with higher concentrations.

Above 10^{-6} M 5-hydroxytryptamine the relative increase of the tone became larger. At 10^{-5} M the effect was almost all increase in tone while the rhythmical excursions of the heart were very small. The ventricle was often arrested in systole. When the increase in tone is plotted as a function of log concentration a really large increase in the slope starts rather suddenly at about 3×10^{-6} M (Fig. 4). At these high concentrations the frequency of beat often doubled or trebled.

Thus, there appears to be two distinct responses of the *Venus* heart to 5-hydroxytryptamine. One, at low to moderate concentrations, is dominated by a positive inotropic effect. Tone is relatively unimportant and frequency changes are not dependable. The other response, evoked by high concentrations, is dominated by a large increase in muscle tone. The inotropic effect no longer followed the relationship implied by the sigmoid curve found at lower concentrations. The frequency always increases noticeably.

A measure of the 5-hydroxytryptamine effect at lower concentrations is the difference, in mm., between the amplitude of beat before and after the addition of a dose (b–a in the inset of Fig. 4). It is interesting that, from threshold to about 10^{-6} M 5-hydroxytryptamine, the plot of this effect against log concentration was the expected sigmoid curve. Above 10^{-6} M, when the tone also suddenly increased greatly, the points ceased to follow this relationship (Fig. 4). The action of 5-hydroxytryptamine in concentrations below 10^{-6} M is dealt with elsewhere (Greenberg, 1960). The present work concerns the effects of doses greater than 3×10^{-6} M.

Brom LSD (10^{-5} g./ml.), given 20 min. previously, antagonized the action of moderately high doses of 5-hydroxytryptamine ($3–10\times10^{-7}$ M). This blockade was usually surmountable by concentrations of

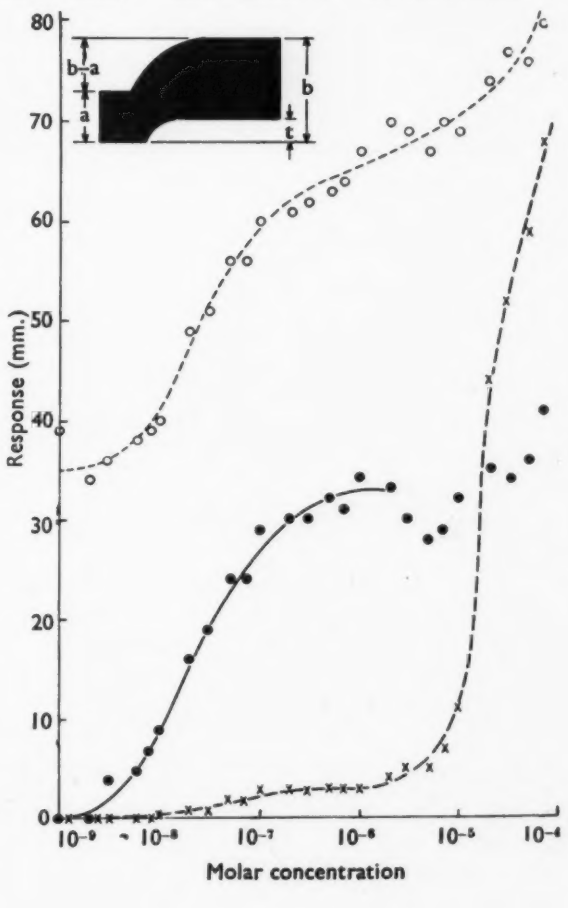


FIG. 4.—The effects of increasing concentrations of 5-hydroxytryptamine on the performance of the isolated *Venus* heart preparation (see Fig. 4). (**X**) indicates the amount of tone (t) in the response. (**O**) indicates the final amplitude (b) of the response. (●) represents the difference (b–a) between the final (b) and initial (a) amplitudes. (t), (b), (a), and (b–a) are explained in the inset, which is a diagram of a 5-hydroxytryptamine response.

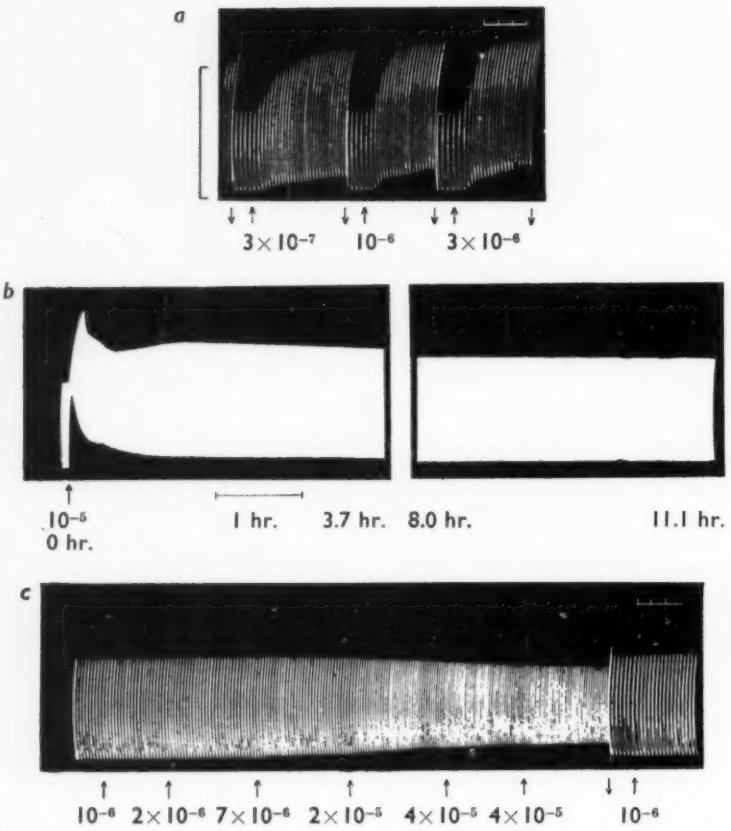


FIG. 5.—Tachyphylaxis of a *Venus* heart preparation to 5-hydroxytryptamine. (*a*) Three standard doses of 5-hydroxytryptamine (5HT) successively washed out (indicated by down-pointed arrow). (*b*) Record made at slow drum speed (Time scale : 1 hr.) showing the secondary decrease in amplitude following a large dose of 5HT. (*c*) Large doses of 5HT now added to tachyphylactic preparation. (*a*), (*b*), and (*c*) are consecutive records from the same preparation. Benzoquinonium chloride (10^{-5} g./ml.) added after every washing. $Dosa_{5}$: moles/litre. Tension: 500 mg. Temperature: 15°. Time scale for (*a*) and (*c*) : 30 sec. Amplitude scale: 5 cm.

5-hydroxytryptamine greater than 10^{-6} M or by smaller doses if the preparation was a sensitive one. Often, the *Venus* heart was excited by 10^{-5} g./ml. Brom LSD (see Greenberg, 1960). Increased time of application or increased dose resulted in substantial excitation. Consequently, Brom LSD is not useful as an inhibitor of high 5-hydroxytryptamine doses.

Tachyphylaxis to 5-Hydroxytryptamine.—When 10^{-5} M 5-hydroxytryptamine was present in the bath the tone of the heart increased greatly. If the dose was not washed out, this effect slowly diminished. After from 4 to 12 hr. the ventricle was beating almost normally (Fig. 5b). The tone and amplitude may be larger than before the application of the large dose of 5-hydroxytryptamine, but usually not as large as it is in response to even moderate concentrations (10^{-9} to 10^{-7} M). Such a preparation was then tachyphylactic. It did not respond to the further addition of increasing doses of 5-hydroxytryptamine up to 10^{-5} M (Fig. 5c). It also did not respond to 5-hydroxytryptamine analogues, such as tryptamine and bufotenine (Fig. 6a). Furthermore, hearts could be made tachyphylactic to tryptamine (Fig. 8a), bufotenine, or 5-hydroxy-α-methyltryptamine (Fig. 6b), and afterwards these hearts will be unresponsive to 5-hydroxytryptamine as well as to the desensitizing drugs. On the other hand, the catechol amines still excited desensitized hearts in their characteristic manner, although the magnitude of the response may be reduced (Fig. 6c).

Isolated *Venus* hearts which have been left untreated for 12 hr. responded to *all* 5-hydroxytryptamine concentrations in the same manner as fresh preparations. Thus, tachyphylaxis does not depend upon the age of the preparation. It was not possible to restore the sensitivity of the tachyphylactic heart to 5-hydroxytryptamine even after prolonged washing. Specific tachyphylaxis to 5-hydroxytryptamine has been described in guinea-pig ileum and other mammalian tissues (Gaddum, 1953; Gaddum and Hameed, 1954). Tachyphylaxis, in these tissues, occurs relatively rapidly and is reversible.

High 5-Hydroxytryptamine Concentrations after Tachyphylaxis.—Desensitized hearts were insensitive to doses of 5-hydroxytryptamine up to 10^{-5} M. However, when challenged with 2×10^{-5} M, the beat of such a heart *decreased* in amplitude. With increasing doses, up to 10^{-4} M (which caused the heart to be arrested immediately in systole), responses were produced which were almost identical with those to adrenaline and noradrenaline (Fig. 7). These catechol amine-like effects were also elicited by 5-hydroxytryptamine if the heart had been made tachyphylactic by one of its analogues (Fig. 8*a*). Furthermore, the decrease in amplitude at about

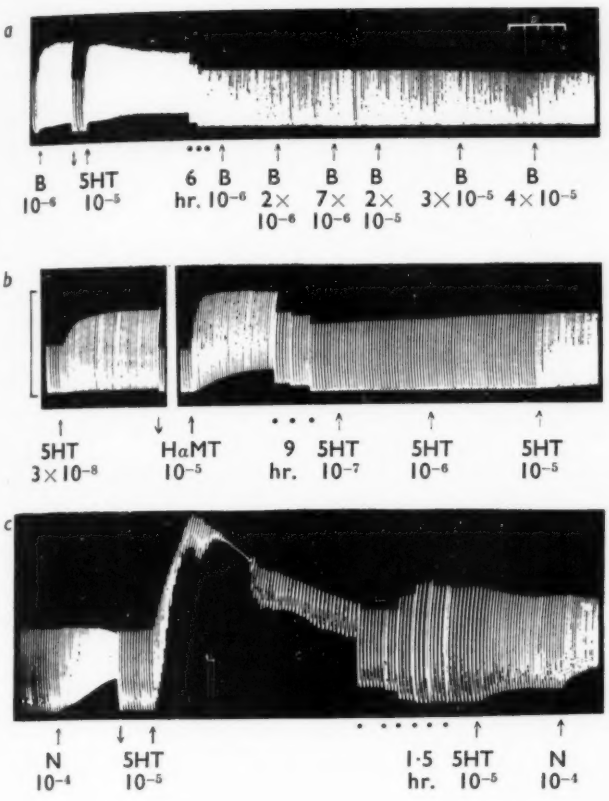


FIG. 6.—Responses of the tachyphylactic *Venus* heart preparation. (*a*) After a standard dose of bufotenine (B), the heart is made tachyphylactic to 5-hydroxytryptamine (5HT). Bufotenine, subsequently, has no effect. (*b*) After a standard dose of 5HT the heart is made tachyphylactic to 5-hydroxy-α-methyltryptamine (HαMT). Subsequent doses of 5HT are ineffective. (*c*) Tachyphylaxis produced by 5HT. The noradrenaline (N) response is relatively unaffected. (●) indicates stopping of drum. The total time required for tachyphylaxis is indicated. Washing was at the downward-pointing arrows. Benzoquinonium chloride (10^{-5} g./ml.) was added to the sea water after each washing. Doses: moles/litre. Tension: 500 mg. Temperature: 15°. Time scale: 30 sec. Amplitude scale: 4 cm.

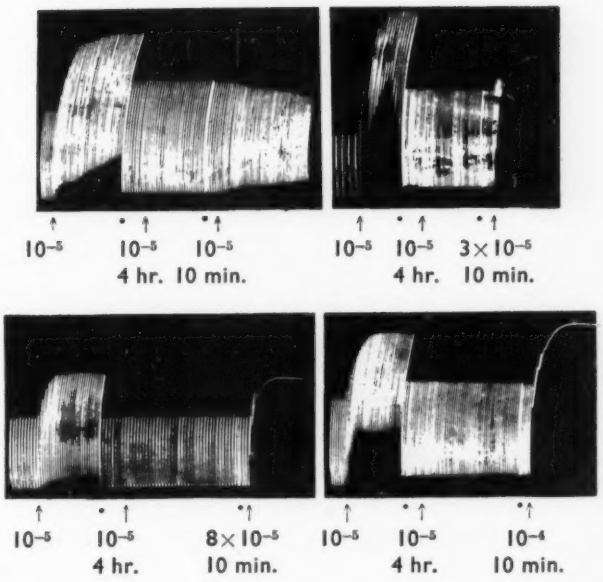
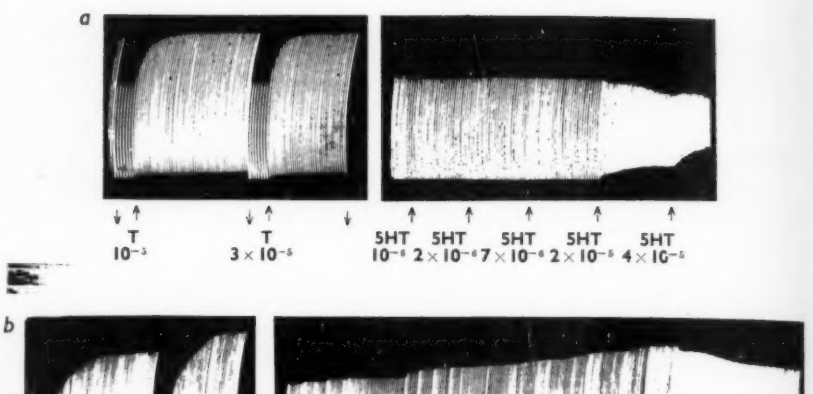


FIG. 7.—Catechol-amine-like responses (see Fig. 1) produced by 5-hydroxytryptamine (5HT) on tachyphylactic *Venus* heart preparations. (●) indicates that the drums were stopped for the time shown. Doses: moles/litre. Tension: 500 mg. Temperature: 15°.

2×10^{-5} M occurred even if the positive inotropic effect, due to a high dose of a tryptamine analogue had not disappeared (Fig. 8b). Such a decrease in amplitude after 10^{-5} M 5-hydroxytryptamine occurred when an exciting dose of Brom LSD (10^{-5} mg./ml.) was left in the bath for 7 hr. (Fig. 8c). The similarity of responses and concentrations suggests that the 5-hydroxytryptamine is acting at the same sites as the catechol amines.

It has been impossible to obtain this distinctive series of responses with either tryptamine or bufotenine. Tryptamine, at 10^{-3} M, after tachyphylaxis had, in a few experiments, produced effects resembling the *n*-alkylamine effect. Bufotenine, at least up to 4×10^{-5} M, produced only a slight decrease in amplitude with a small increase in tone on a tachyphylactic heart.



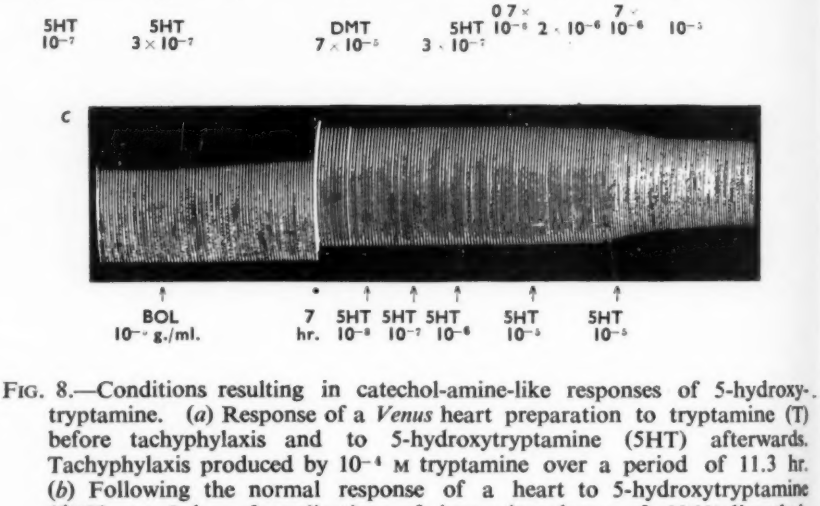


FIG. 8.—Conditions resulting in catechol-amine-like responses of 5-hydroxytryptamine. (*a*) Response of a *Venus* heart preparation to tryptamine (T) before tachyphylaxis and to 5-hydroxytryptamine (5HT) afterwards. Tachyphylaxis produced by 10^{-4} M tryptamine over a period of 11.3 hr. (*b*) Following the normal response of a heart to 5-hydroxytryptamine (5HT) are 8 hr. of application of increasing doses of *N'N'*-dimethyltryptamine (DMT), the last of which is shown. Successive doses of 5HT are then added. Note the small positive inotropic effects, up to 2×10^{-5} M 5HT, indicating the absence of tachyphylaxis. (●) indicates that drum was stopped for 7 min. (*c*) Catechol-amine-like effect of 5HT after prolonged treatment with 2-brom-(+)-lysergic acid diethylamide (BOL). Doses: moles/litre. Tension: 1,000 mg. Temperature: 15°.

The Effect of 5-Hydroxytryptamine at High Temperatures.—High bath temperatures reduced the response of the heart to 5-hydroxytryptamine. At 30° to 35° the muscle lengthened and the addition of doses of 5-hydroxytryptamine, up to 10^{-7} M, had very little effect. When 10^{-5} M 5-hydroxytryptamine was then added there followed the decrease in amplitude which had been associated both with 5-hydroxytryptamine after tachyphylaxis and with threshold doses of catechol amines. When the bath is cooled to 15° the usual 5-hydroxytryptamine effect, at 10^{-5} M, with its high tone and small rhythmical excursions, is unmasked. Thus, it was possible to demonstrate the catechol-amine-like response to high concentrations of 5-hydroxytryptamine without desensitizing the heart to 5-hydroxytryptamine.

DISCUSSION

A *Venus* heart preparation may be either excited or depressed by the catechol amines, depending upon the dose. Although previous workers have reported diverse effects for adrenaline and noradrenaline on lamellibranch hearts (Krijgsman and Divaris, 1955 ; Fänge, 1955 ; Gaddum and Paasonen, 1955), only for *Anodonta cygnea* is there a suggestion of more than one mode of action for

these compounds. Fänge (1955) mentions not only a negative inotropic effect, but also immediate systolic arrest at a ten-fold higher concentration. Similarly, Ten Cate (1923) and Hendrickx (1945) noted an enfeeblement of *Anodonta* heart beat prior to systolic arrest.

It seems possible that the hearts of some animals, such as *Amblema peruviana* (Motley, 1934), *Cardium edule* (Gaddum and Paasonen, 1955), and the oyster (Jullien, 1936), for which a depression by catechol amines has been reported, are especially sensitive to the negative inotropic effect of these compounds. Presumably, higher doses would evoke the excitation which has been seen in *Venus* and others. One would also expect to produce, with lower concentrations than have been used in the past, a decrease in amplitude from clam heart preparations which have been noted to be stimulated by the catechol amines.

The exciter effect of adrenaline and noradrenaline on the *Venus* heart preparation was not only of lower potency than that of 5-hydroxytryptamine below 10^{-6} M but also qualitatively different. Welsh made this distinction between the two excitatory actions in 1953.

While other lamellibranch hearts are, in general, between 1,000 and 10,000 times more sensitive to 5-hydroxytryptamine than to adrenaline, only *Cardium edule* (Gaddum and Paasonen, 1955) and *Anodonta cygnea* (Fänge, 1955) show any qualitative distinction between the two exciter effects.

The difference, on *Venus* heart, between the actions of catechol amine and 5-hydroxytryptamine suggests that these actions originate from different sites in the tissue. This contention is supported by two sorts of evidence. First, when a preparation is insensitive to low or moderate doses of 5-hydroxytryptamine, due to tachyphylaxis or high bath temperatures (30° to 35°), it will display the various effects associated with the catechol amines if challenged either by high doses of these amines or by 5-hydroxytryptamine. Second, Brom LSD (10^{-5} g./ml.), which inhibits 5-hydroxytryptamine, has little effect on the response to adrenaline. Brom LSD also produces tachyphylaxis; this has been observed only when it was exciting the heart. Therefore, it is impossible to say whether the catechol-amine-like effect would also follow an inhibition of 5-hydroxytryptamine when not preceded by excitation. However, Brom LSD does not antagonize the effect of 5-hydroxytryptamine seen in the desensitized heart; this strengthens the presumption that this effect does not occur as a result of excitation at the usual 5-hydroxytryptamine site.

It would be interesting to know whether there is in *Venus*, a physiological basis for the response to catechol amines or whether it is merely an unspecific effect of toxicological significance only. There is some evidence which suggests that a physiological role exists.

As far as has been studied, there seem to be relatively exacting structural requirements for molecules producing this effect. Thus, in contrast to 5-hydroxytryptamine, tryptamine is inactive. Phenylethylamine analogues which either lack hydroxyl groups or have methoxy groups (mescaline) in any two of the three hydroxyl positions in noradrenaline are similarly ineffective. The activity of dopamine, which has no side chain hydroxyl, suggests that only the two phenolic groups of noradrenaline are necessary for the production of the characteristic response. Whether the hydroxyl on the side chain would suffice, instead of that in the 3-position of the nucleus, must be established by testing *p*-hydroxyphenylethanolamine (octopamine). This prospect is interesting since octopamine is a natural product found abundantly in the posterior salivary glands of *Octopus vulgaris* (Bacq, Fischer and Ghiretti, 1952). The *n*-alkylamines and histamine, while excitatory, each have actions which are distinctive. The structure-activity relations of the response to the catechol amine have not been worked out. However, present information indicates that a structurally specific receptor in the cell is involved.

The responses of the tachyphylactic or overwarmed *Venus* heart to 5-hydroxytryptamine are duplicated seasonally in nature. During the summer months (late June to August) the threshold of the ventricle to 5-hydroxytryptamine may increase by ten-fold or more. In fact, normally beating hearts sometimes cannot be excited until concentrations of 10^{-6} M are reached, and then the response is small. Furthermore, in the summer, a *decrease* in amplitude of beat in response to low 5-hydroxytryptamine doses (about 10^{-8}) has occasionally been observed (B. M. Twarog ; M. K. Paasonen ; personal communications). The decrease is not acetycholine-like (see Welsh and Taub, 1948) ; the rate of beat increases and the response resembles, in fact, the effect of noradrenaline between 10^{-5} and 2×10^{-5} M. The production of the catechol amine effect by the normal excitatory neurohumour, 5-hydroxytryptamine, under naturally induced circumstances thus occurs. This suggests that the series of responses evoked experimentally by the catechol amines are physiological. No explanation for the increase of threshold of the 5-hydroxytryptamine effect in the summer is at hand. The possibility of a

relationship between summer and high temperature insensitivity and the irreversible tachyphylaxis with its slow onset is obvious and intriguing, but it has not been explored.

Finally, catechol amines have been found in the ganglia of *Venus mercenaria* (Welsh, personal communication). This strongly suggests that the catechol amines, themselves, have a role in the normal functioning of *Venus* and, more specifically, in the seasonal variation seen in the performance of the heart.

A practical result of the present work is that, as a consequence of the response of the heart to high 5-hydroxytryptamine concentrations, two tests for the specificity of action of excitatory compounds become available. The first involves the assumption that when the heart preparation is tachyphylactic to 5-hydroxytryptamine the receptors are blocked and there cannot subsequently be a response to any compound which initiates its actions by attachment to this receptor. Conversely, it is to be expected, and has been shown, that long applications of high concentrations of specifically acting compounds will also result in insensitivity to 5-hydroxytryptamine. Gaddum (1953) first used tachyphylaxis to 5-hydroxytryptamine in this way to demonstrate the existence of specific tryptamine receptors in the guinea-pig ileum.

The application of 2×10^{-5} M 5-hydroxytryptamine to the tachyphylactic *Venus* heart preparation results in a *decrease* in amplitude ; a ten-fold higher dose causes systolic arrest. This phenomenon constitutes the second means of distinguishing 5-hydroxytryptamine-like substances from other excitatory agents. It is especially useful in studies of test compounds which act irreversibly and cause tachyphylaxis, with a slow onset, after high doses.

It is a privilege to acknowledge the advice and encouragement offered by Professor J. H. Welsh during the course of this investigation.

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STRUCTURE-ACTIVITY RELATIONSHIP OF TRYPTAMINE ANALOGUES ON THE HEART OF *VENUS MERCENARIA*

BY

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A number of tryptamine analogues and other exciter agents have been tested on the heart of *Venus mercenaria*. The method of estimation of potency, especially for irreversibly acting compounds, is discussed. Specificity of action with respect to the site of action of 5-hydroxytryptamine is defined experimentally. The specific activity of tyramine and phenethylamine and the non-specific excitatory action of indole and skatole indicate that the indole ring is neither necessary nor sufficient for 5-hydroxytryptamine-like activity. Tryptamine analogues differ in mode of action as well as potency. Congeners without a 5-hydroxyl group tend to act more slowly and irreversibly as well as less strongly than 5-hydroxytryptamine. Methyl substitution also increases the time of action and difficulty of reversal. However, the potency of such compounds may be increased or decreased depending upon the position of substitution and the presence of the 5-hydroxyl group. The relations between structure and potency and mode of action are discussed. Suggestions are made concerning the effective conformation of the 5-hydroxytryptamine molecule and the nature of its receptor.

5-Hydroxytryptamine might be a chemical transmitter in mollusca (Bacq, Fischer and Ghiretti, 1952 ; Welsh, 1953, 1954 ; Twarog, 1954 ; Hill, 1958). The evidence supporting this contention has recently been summarized by Welsh (1957).

The structure-activity relations of 5-hydroxytryptamine have been explored only rarely in preparations of mollusc (Marczyński, 1959), although isolated mammalian organs have often been used for this purpose (Page, 1958 ; Barlow and Khan, 1959a, b ; Vane, 1959). The great sensitivity to 5-hydroxytryptamine of the isolated ventricle of the clam, *Venus mercenaria,* makes it an excellent preparation for such a study.

In the course of this work a number of indole analogues and other excitor agents have been tested on the *Venus* heart preparation. Some suggestions are made regarding the binding sites and effective conformation of the 5-hydroxytryptamine molecule as well as the shape of its receptor.

METHODS

Preparation.—Hearts of *Venus mercenaria* (the quahog) were removed from the animals by the method of Welsh and Taub (1948). The hearts were set up in a 10 ml. perfusion bath at 15°. Treatment of the hearts in the bath, drug administration and recording were as previously described (Greenberg, 1960).

The Effect and its Measurement.—5-Hydroxytryptamine increases the force of contraction of the *Venus* heart and, at high concentrations, augments the tone as well. The measure of exciter effect used in this study is the difference, in mm., between the amplitude of beat before and after the response to an agent. Any increase of tone which occurs is included in the measurement of the final amplitude. A given effect of 5-hydroxytryptamine, or of any other compound tested, was considered completed, and therefore measurable, when there was no longer any change of amplitude following administration of a given dose ; that is, when the drug bound to the receptors was in equilibrium with the drug in the perfusion fluid. Consequently, time of action varies with dose, drug and preparation. However, the time of action for a given dose of any drug, relative to that of 5-hydroxytryptamine, is approximately the same in every preparation.

The log dose-response curve for 5-hydroxytryptamine is sigmoid between about 10^{-9} and 10^{-6} M (Greenberg, 1960). Between about 10^{-6} M and 3×10^{-7} M the curve is nearly linear and the increase in tone is never more than 15% of the response. In the experiments to be described, concentrations of 5-hydroxytryptamine above 3×10^{-7} M were rarely used.

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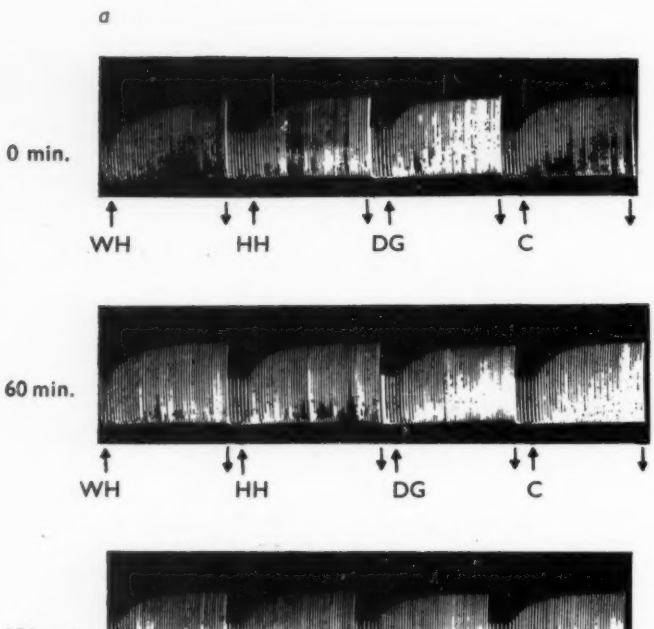


FIG. 1.—Absence of amine oxidase activity in *Venus* heart. (a) The effect, on a heart, of 3×10^{-8} M 5-hydroxytryptamine incubated with whole heart (WH), homogenized heart (HH) and homogenized digestive gland (DG) at room temperature. (C) is the untreated control dose of 5-hydroxytryptamine. Length of incubation period is indicated. (b) Ineffectiveness of iproniazid (Ip) in enhancing the effect of 5-hydroxytryptamine (5HT). (●) indicates that the drum was stopped for 1·5 hr. Washing is at downward-pointing arrows. Benzoquinonium chloride (10^{-5} g./ml.) added to bath after each washing. Dosage: moles/l. in the bath. Tension: 500 mg. Bath temperature: 15°.

The potency of the various compounds tested is expressed as the equiactive molar ratio. 5-Hydroxytryptamine is the standard and has a value of 1.0. The ratios are obtained directly from the log dose-response curves of 5-hydroxytryptamine and the test analogue from the same preparation. The points for these curves, except in the special cases mentioned below, are obtained by adding, and then washing out, successively larger doses of the agent. When the relative potency is independent of concentration, these curves are parallel. Often, however, they are not parallel. In such instances the estimation of the equiactive molar ratio is made at the inflection point of the curve of the test compound. In a few instances individual responses of the test analogue were matched by equiactive doses of 5-hydroxytryptamine to obtain the equiactive molar ratio.

Some tryptamine congeners produce irreversible effects so that relative potency is dependent upon concentration. The points for the dose-response curves of such compounds cannot be obtained by washing out a series of successively larger doses. Estimation of potency by matching is also impossible. Therefore in such cases the following method was employed to obtain the equiactive molar ratio. A low concentration of the irreversibly acting drug was added to the bath. After 20 min. with no response, or after a completed response, another dose-increment was added to the bath without washing to make the next concentration in the series, and so on. For any dose, the initial amplitude taken for computation was that prior to the first small dose.

This method of demonstrating the dose-response relationships of slow, irreversibly acting compounds is justified as follows. Any amine oxidase which is present in *Venus* heart and liver tissue is pharmacologically undemonstrable. 5-Hydroxytryptamine incubated with whole heart, homogenized heart, or homogenized digestive gland in no instance decreased in potency over 3 hr. incubation at room temperature (Fig. 1a). Also, pretreatment of hearts with an amine oxidase inhibitor, iproniazid phosphate (Hoffmann-LaRoche) (10^{-4} M), for 1 hr. has no effect on the response to 5-hydroxytryptamine (Fig. 1b). Therefore, there is no need to suppose that the drugs are slowly destroyed during the course of the experiment. Finally, 5-hydroxytryptamine dose-response curves produced on the same heart are similar, whether or not the previous dose was washed out (Fig. 4).

Specificity.—Three tests have been applied to determine whether an exciter agent is acting at the 5-hydroxytryptamine site.

(1) The only effective inhibitor of 5-hydroxytryptamine on the *Venus* heart is (+)-2-bromolysergic acid diethylamide (Brom LSD). A concentration of 10^{-5} g./ml. of this compound present in the bath for 20 min. will usually completely inhibit the action of even high doses of 5-hydroxytryptamine (Welsh and McCoy, 1957). On the other hand, adrenaline, noradrenaline, histamine and n-alkylamines excite the *Venus* heart, but are not antagonized by Brom LSD (Greenberg, 1960).

(2) Drugs which are rendered ineffective by tachyphylaxis of the preparation to 5-hydroxytryptamine are thought to act specifically (Greenberg, 1960). Such compounds in high concentrations can, furthermore, make the heart insensitive to 5-hydroxytryptamine or other specifically acting analogues.

(3) When specific tryptamine analogues in concentrations greater than 10^{-6} M have been left on the heart for over 2 hr., the addition of 10^{-5} M or 2×10^{-5} M 5-hydroxytryptamine will cause a decrease in amplitude. This is usually accompanied by a small increase in tone and a chronotropic effect characteristic of the action of the catechol amines on this preparation (Greenberg, 1960).

Drugs Used.—The compounds used in this study were 5-hydroxytryptamine creatinine sulphate, gramine, 5-hydroxytryptophan, indol-3-ylacetic acid, indol-3-ylpropionic acid (Nutritional Biochemicals); tryptamine [3-(2-aminoethyl)indole] hydrochloride, 5-hydroxyindol-3-ylacetic acid (Mann Research Laboratories); (+)-lysergic acid diethylamide, (+)-2-bromolysergic acid diethylamide (Sandoz Pharmaceuticals); indole, skatole (3-methylindole), phenethylamine (Eastman Kodak Co.); tyramine (*p*-hydroxyphenethylamine) (Abbott Laboratories); tryptophan (California Foundation for Biochemical Research); bufotenine [5-hydroxy-*N'N'*-dimethyltryptamine; 3-(2-dimethylaminoethyl)-5-hydroxyindole], α-methyltryptamine [3-(2-aminopropyl)indole], 5-hydroxy-α-methyltryptamine [3-(2-aminopropyl)-5-hydroxyindole] creatinine sulphate, *N'*-methyltryptamine [3-(2-methylaminoethyl)-indole], *N'N'*-dimethyltryptamine [3-(2-dimethylaminoethyl)indole], *N'*-ethyltryptamine [3-(2-ethylaminoethyl)indole], *N'N'*-diethyltryptamine [3-(2-diethylaminoethyl)indole], 3-(3-dimethylaminopropyl)indole (Upjohn); 5-methoxy-2-methyltryptamine [3-(2-aminoethyl)-5-methoxy-2-methylindole] hydrochloride, 5-hydroxy-2-methyltryptamine [3-(2-aminoethyl)-5-hydroxy-2-methylindole] hydrochloride (Merck Sharp and Dohme); benzoquinonium chloride (Mytolon) (Sterling-Winthrop).

Drugs were made up in M/10 to M/1,000 stock solutions and diluted with distilled or sea water. All doses are expressed as molar concentrations in the organ bath.

RESULTS

The Effects of Phenethylamine and Tyramine.—The action of phenethylamine is a positive inotropic effect, the threshold concentration being about 10^{-5} M. At 10^{-4} M the increase is large and takes about 75 min. to develop completely. Its potency compared with that of 5-hydroxytryptamine is small; the equiactive molar ratio is 2,000. Tyramine, the *p*-hydroxy derivative of phenethylamine, is about 4 times more potent and 1.5 times faster acting than the latter. The actions of both compounds are antagonized by Brom LSD (10^{-5} g./ml.), indicating that they act at the same site as 5-hydroxytryptamine (Fig. 2a, b).

The Effects of Indole and Skatole.—Indole and its 3-methyl derivative, skatole, elicit a positive inotropic response from the *Venus* heart. Threshold is about 3×10^{-6} M. The effect at 10^{-4} M is large and equal to that of 3×10^{-5} M 5-hydroxytryptamine; however, this increase is not blocked by Brom LSD (Fig. 2c, d). Consequently, the action of the indole ring is non-specific.

Close Congeners of 5-Hydroxytryptamine.—The compounds to be discussed here are all closely related in structure to 5-hydroxytryptamine. The actions have, in most cases, been shown to be specific by one or more of the tests previously mentioned. The experimental results are listed in Table I.

Group A.—Tryptamine is about 10 times less active than 5-hydroxytryptamine. Furthermore, the effect of an equiactive dose of tryptamine takes about twice as long to develop (Fig. 3a) and to wash out. The slow recovery from the response is especially evident at higher concentrations. The log dose-response curves for 5-hydroxytryptamine and tryptamine are usually parallel or only slightly divergent (Fig. 4a).

Group B.—Tryptamine analogues with no 5-hydroxyl group, and with methyl or ethyl groups substituted on either the nitrogen or carbon atoms of the side-chain, produce a response which is in certain respects different from that to 5-hydroxytryptamine.

Firstly, the effects are very slow to develop. For example, the response to 3×10^{-5} M *N'*-methyltryptamine may take 5 min. to become noticeable and between 30 and 40 min. to be completed (Fig. 3a). At higher doses this time is often longer.

Secondly, the response is irreversible. While prolonged washing decreases the effect slightly, the original amplitude is never regained. Due to the irreversibility of these compounds, points for dose-response curves were found by adding successively larger doses without interposed washing, as described under methods.

Thirdly, the relationship between concentration and effect of the *N'*-alkyl-substituted compounds is unlike that of 5-hydroxytryptamine. Threshold

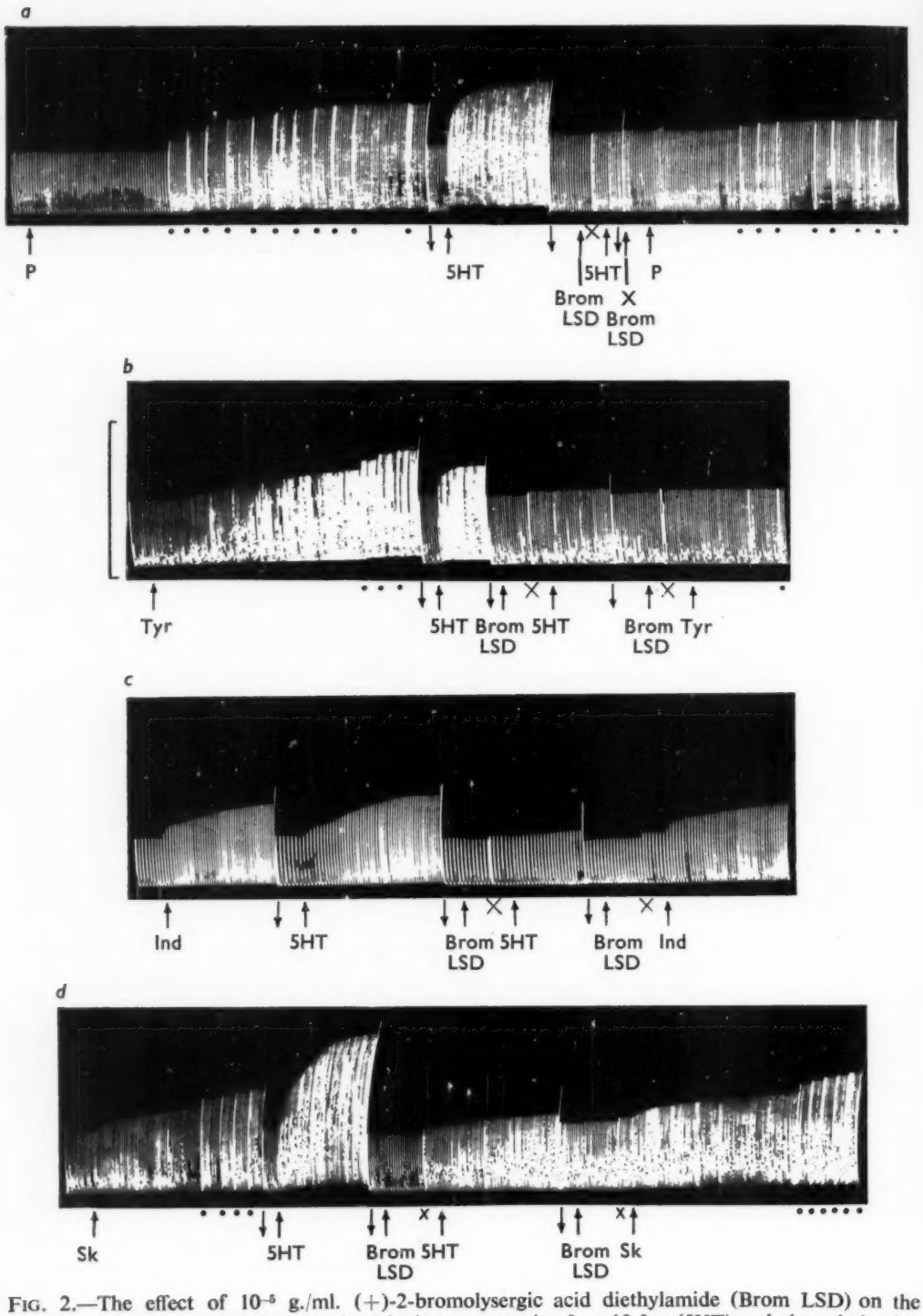
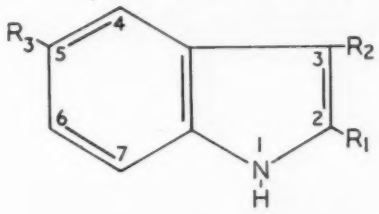


FIG. 2.—The effect of 10^{-5} g./ml. (+)-2-bromolysergic acid diethylamide (Brom LSD) on the responses of the *Venus* heart to (a) 5-hydroxytryptamine 3×10^{-7} M (5HT) and phenethylamine 10^{-4} M (P); (b) 5-hydroxytryptamine 3×10^{-7} M (5HT) and tyramine 10^{-7} M (Tyr); (c) 5-hydroxytryptamine 3×10^{-8} M (5HT) and indole 10^{-4} M (Ind); (d) 5-hydroxytryptamine 10^{-7} M (5HT) and skatole 10^{-4} M (Sk). Following each dose of Brom LSD the drum was stopped (at X) for 20 min. (●) indicates drum was stopped; total elapsed time is 60 min. in (a) and (b) and 40 min. in (d). Downward-pointing arrows indicate washing. Benzoquinonium chloride (10^{-5} g./ml.) added to bath after washing. Tension: 1,000 mg. Temperature: 15°. Heart rate: 10/min. Amplitude scale: 6 cm.

TABLE I

RELATIVE POTENCIES OF TRYPTAMINE ANALOGUES ON *VENUS* HEART

The equiactive molar ratio of 5-hydroxytryptamine is taken as unity. N.E. means that no effect could be elicited from the preparation.



Group	Compound	R_1	R_2	R_3	Equiactive Molar Ratio: Individual Values	Equiactive Molar Ratio: Mean
A	Tryptamine	H	$-CH_2-CH_2-NH_2$	H	15, 14, 13, 11, 10, 6, 6, 4	9·9
B	*N'*-Methyltryptamine	H	$-CH_2-CH_2-NH(CH_3)$	H	6, 5, 4, 4, 3·3, 2·2, 1·4	3·7
	N'-Ethyltryptamine	H	$-CH_2-CH_2-NH(C_2H_5)$	H	15, 14, 10, 10, 7, 4·5, 3	9·1
	N'N'-Dimethyltryptamine	H	$-CH_2-CH_2-N(CH_3)_2$	H	30, 15, 12·5, 10, 4, 2, 1·7	10·7
	N'N'-Diethyltryptamine	H	$-CH_2CH_2-N(C_2H_5)_2$	H	12, 12, 10, 10, 8·6, 1·7, 1	7·9
	α-Methyltryptamine	H	$-CH_2-CH(CH_3)-NH_2$	H	10, 8·7, 7	8·6
C	Bufotenine	H	$-CH_2-CH_2-N(CH_3)_2$	–OH	0·04, 0·03, 0·025, 0·017	0·028
	5-Hydroxy-α-methyltryptamine	H	$-CH_2-CH(CH_3)-NH_2$	–OH	6, 6	6
D	5-Hydroxy-2-methyltryptamine	$-CH_3$	$-CH_2-CH_2-NH_2$	–OH	60, 35, 30, 17, 15	31·4
	5-Methoxy-2-methyltryptamine	$-CH_3$	$-CH_2-CH_2-NH_2$	$-OCH_3$	65, 50, 35, 25	43·8
E	Gramine	H	$-CH_2-N(CH_3)_2$	H	N.E., N.E., N.E., 10,000, 7,000, 170	—
	N'N'-Dimethyltryptamine	H	$-CH_2-CH_2-N(CH_3)_2$	H	30, 15, 12·5, 10, 4, 2, 1·7	10·7
	3-(3-Dimethylaminopropyl)indole	H	$-CH_2-CH_2-CH_2-N(CH_3)_2$	H	3,300, 1,700, 1,000	2,000

is usually 10^{-9} M or 3×10^{-9} M. In 82% of the experiments the response to low concentrations (10^{-9} to 10^{-8} M) of the *N'*-alkyltryptamines was equal to, or greater than, that to the same dose of 5-hydroxytryptamine. The maximum effect was achieved at 10^{-5} to 10^{-4} M. However, both the inotropic effect and the increased tone resulting from this maximal response are only equivalent to those of 3×10^{-8} M to 3×10^{-7} M 5-hydroxytryptamine.

The log dose-response curves which result vary greatly in shape (Fig. 4). In general the curves are sigmoid with a lower limb which may cross the 5-hydroxytryptamine curve of the same preparation (Fig. 4b). The middle, steeply-rising, portion of the *N'*-alkyltryptamine curves starts at about 10^{-7} M and is usually divergent from that of 5-hydroxytryptamine. On the other hand, with *N'N'*-diethyl- or *N'*-ethyl-tryptamine this portion of the curves is often parallel to the standard (Fig. 4c). In about a third to a half of the experiments, once a response at low concentrations was established, further increases in dose (up to about 10^{-7} M) produced either small or negligible increases in amplitude. The log dose-response curves which resulted were biphasic with a

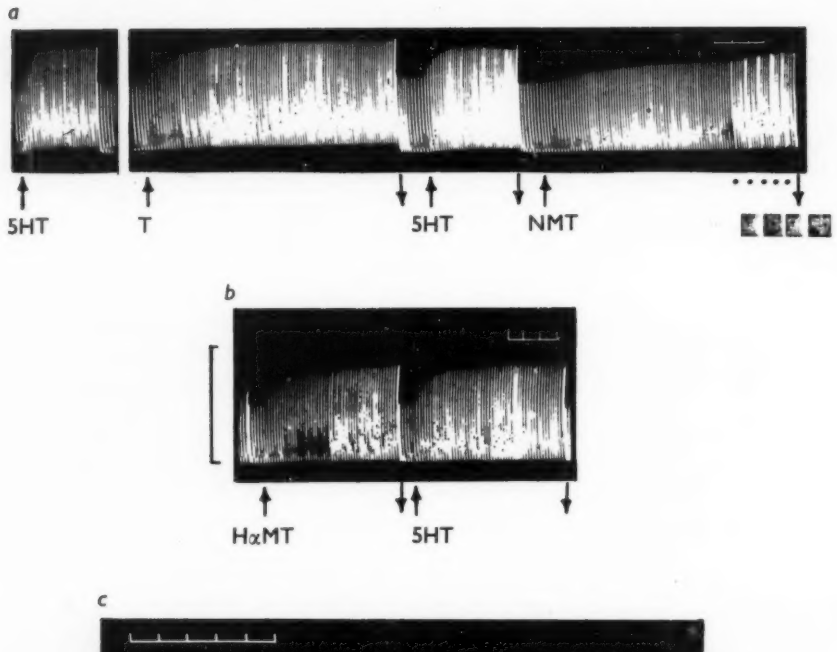


FIG. 3.—Comparison of velocities of *Venus* heart preparations in response to (a) 5-hydroxytryptamine 3×10^{-7} M (5HT), tyramine 3×10^{-6} M (T), 5-hydroxytryptamine 10^{-7} M (5HT) and *N'*-methyltryptamine 10^{-8} M (NMT); (b) 5-hydroxy-α-methyltryptamine 10^{-7} M (HαMT) and 5-hydroxytryptamine 2×10^{-8} M (5HT); (c) bufotenine 10^{-8} M (B) and 5-hydroxytryptamine 10^{-7} M (5HT). At (●) the drum was stopped for 5 min. Washing is at downward-pointing arrows. Benzoquinonium chloride (10^{-5} g./ml.) added to the bath immediately after washing and 5 min. before succeeding dose. Tension: 500 mg. Temperature: 15°. Time scale: 30 sec. Amplitude: 3 cm.

plateau between about 10^{-8} M and 10^{-7} M (Fig. 4c). Such variation in the shape of the curves, and their divergence from those of 5-hydroxytryptamine, suggest that there should be some uncertainty in potency measurements. This is confirmed by the results shown in Table I. The equiactive molar ratios of α-methyl-, *N'*-ethyl-, *N'N'*-diethyl- and *N'N'*-dimethyl-tryptamine are of the same order of magnitude as that of tryptamine itself. However, *N'*-methyltryptamine has a potency about double that of tryptamine.

Group C.—5-Hydroxy-α-methyltryptamine and bufotenine are the 5-hydroxy analogues of two methyl-substituted amines previously discussed (Group B). The presence of the hydroxyl group results in some marked changes in action. First, the responses are reversible. Second, the speed of action increases greatly.

5-Hydroxy-α-methyltryptamine is not significantly more active than α-methyltryptamine, but bufotenine is about 500 times as active as *N'N'*-dimethyltryptamine.

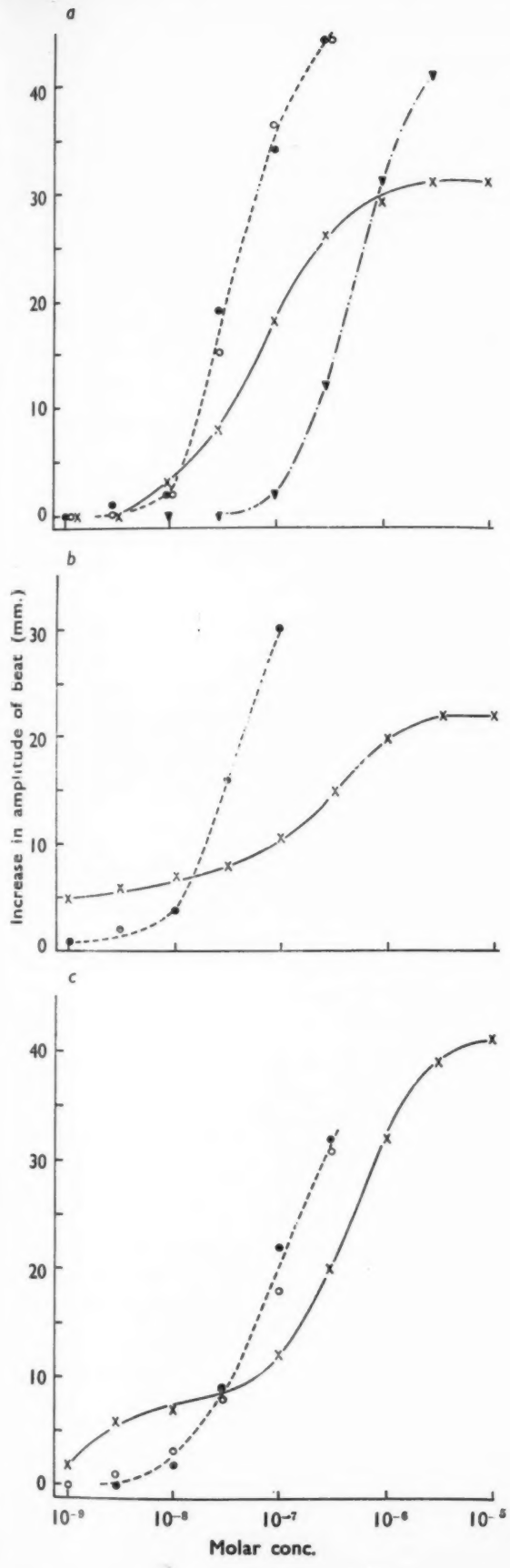


FIG. 4.—Dose-response curves illustrating the variation in the effects of non-hydroxylated tryptamine analogues on three *Venus* heart preparations. The effects of 5-hydroxytryptamine (— — —) are compared with those of: (a) *N'*-methyltryptamine (X——X) and tryptamine (▼—.—▼); (b) *N'N'*-diethyltryptamine (X——X); and (c) *N'*-ethyltryptamine (X——X). In preparations (a) and (c) one set of 5HT points were obtained by washing between successive doses (O) and another by making additions without washing (●).

Of all the compounds which have been tested, 5-hydroxy-α-methyltryptamine produces effects which are most similar to those of 5-hydroxytryptamine. Its time of action is only slightly longer than that of 5-hydroxytryptamine (Fig. 3b). The effects, even of relatively high doses (3×10^{-7} M), are quickly washed out. The log dose-response of the two substances are parallel (Fig. 5a); this is similar to the results with tryptamine.

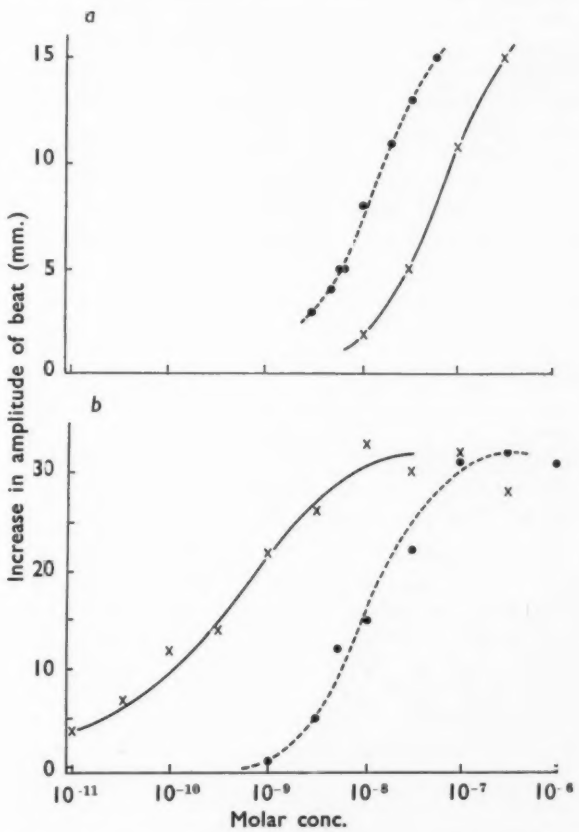


FIG. 5.—Dose-response curves contrasting the effects on *Venus* hearts of 5-hydroxytryptamine (●— — —●) and (a) 5-hydroxy-α-methyltryptamine (X——X) and (b) bufotenine (X——X).

However, compared with tryptamine, 5-hydroxy-α-methyltryptamine is more potent, faster in action, and easier to wash out.

Bufotenine is more potent than 5-hydroxytryptamine. Its structure is intermediate between that of 5-hydroxytryptamine and *N'N'*-dimethyltryptamine, and its mode of action is also intermediate. First, while its effects are not irreversible, the washing time for a similar response is almost twice that of 5-hydroxytryptamine. Second, at threshold or slightly higher concentrations (10^{-11} M to 10^{-9} M), the response to bufotenine may take between 20 and 30 min. to develop and at any concentration it is slower than the equipotent 5-hydroxytryptamine response (Fig. 3c). Third, the log dose-response curve of bufotenine is always less steep than that of 5-hydroxytryptamine (Fig. 5b). Accordingly, the equiactive molar ratio varies with concentration. At threshold it is 1/100. Near the maximum response of bufotenine, at about 3×10^{-8} M, it is approximately 1/10.

Group D.—The compounds of this group have a substituent in the 2-position of the indole ring. This substitution serves to decrease the potency greatly. Thus, 5-hydroxy-2-methyltryptamine is about 30 times less potent than 5-hydroxytryptamine. Its threshold is 3×10^{-7} M. Also, the time of action is slow and the effect is difficult to wash out, especially at high concentrations. 5-Methoxy-2-methyltryptamine is slightly less potent than its 5-hydroxy congener, although its threshold is about the same. It is also far more difficult to wash out. The effects are, in fact, not completely reversible.

Brom LSD might be included here in its capacity as an excitor agent (Greenberg, 1960) with a substituent in the 2-position. The exciter action is extremely undependable. When it occurs, the equiactive molar ratio of a 3×10^{-5} M dose varies between 300 and 30,000 depending upon the preparation. The time of action is slow and the effect is irreversible.

Group E.—The substances included here differ only in the length of the side-chain at the 3-position. In gramine it is one, in *N'N'*-dimethyltryptamine it is two, and in 3-(3-dimethylaminopropyl)indole it is three carbon atoms long. All three compounds have two methyl groups on the amino-nitrogen atom. None has a 5-hydroxyl group in the indole ring.

It is clear that increasing or decreasing the length of the side-chain results in a profound loss of activity (Table I). This loss is greatest with gramine. Gramine is well known as a 5-hydroxytryptamine antagonist in a variety of preparations. It also acts in this way, although weakly, on *Venus* heart. Applied to the heart for an hour, 10^{-4} M gramine decreases the response by more than half. 3-(3-Dimethylaminopropyl)indole is only slightly inhibitory. Its excitatory action at high concentrations usually masks any inhibition which might occur.

No attempt was made to block the action of gramine or 3-(3-dimethylaminopropyl)indole with Brom LSD. Since these two compounds antagonize 5-hydroxytryptamine, it was assumed that they act at the 5-hydroxytryptamine site. However, the specificity of the antagonism has not been demonstrated.

The Effects of Indol-3-yl Acids.—Of the indol-3-yl acids which have been examined, some are in the 5-hydroxytryptamine metabolic pathway and others are not. It might be expected that tryptophan (α-aminoindol-3-ylpropionic acid) and 5-hydroxytryptophan, which are precursors of 5-hydroxytryptamine (Udenfriend, 1958), would be converted by enzymes in the heart. Their effect should then be a slow increase in the amplitude of the heart. In fact, tryptophan is completely inactive up to 10^{-3} M. 5-Hydroxytryptophan excites feebly and occasionally at 10^{-3} M. Its potency relative to 5-hydroxytryptamine is about 100,000.

5-Hydroxyindol-3-ylacetic acid is a product of the enzymic breakdown of 5-hydroxytryptamine (Udenfriend, Wiessbach, and Bogdanski, 1957). This acid does excite feebly at 10^{-4} M. The equiactive molar ratio is 20,000.

Indol-3-ylacetic acid and indol-3-ylpropionic acid are essentially inert although indol-3-ylacetic acid excites at 10^{-3} M. The equiactive molar ratio is over 100,000.

These acids are so feebly active that no attempt was made to antagonize their action with Brom LSD. Indeed, the exciter response to the latter at 2×10^{-5} g./ml. is usually of greater magnitude.

At least two attempts were made, using each of these acids, to block the actions of 5-hydroxytryptamine. None were successful.

Lysergic Acid Diethylamide.—Characteristics encountered in the *N'*-alkyltryptamines are emphasized in lysergic acid diethylamide. The usual threshold concentrations (10^{-16} M–10^{-15} M) often result in a nearly maximal response which, however, takes 4 hr. to be completed (Welsh and McCoy, 1957). The log dose-response curves from such preparations cross, almost perpendicularly, those of 5-hydroxytryptamine (Fig. 6a). Estimation of potency in such cases is meaningless. However, not all hearts respond in this

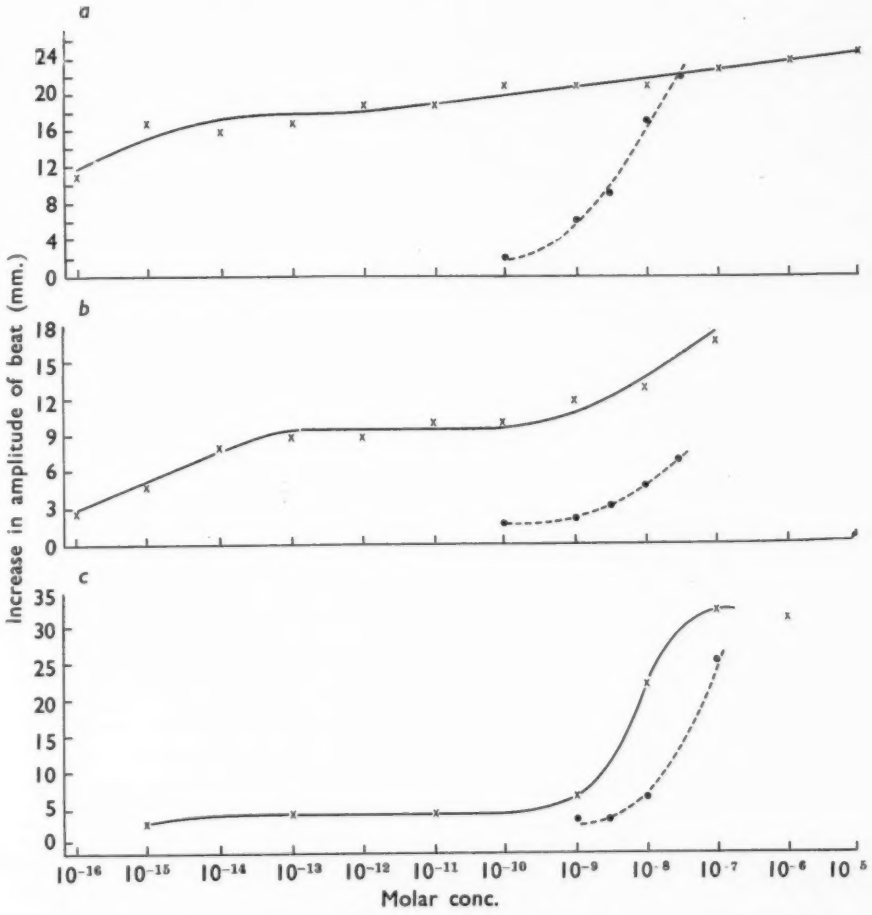


FIG. 6.—Dose-response curves showing variations in the responses of different *Venus* heart preparations to (+)-lysergic acid diethylamide (X——X) and to 5-hydroxytryptamine (● — — — ●).

manner. Some preparations are affected less strongly by low concentrations of lysergic acid diethylamide. The curve in these cases may turn upward at about 10^{-10} M (Fig. 6b). In two experiments, there was almost complete insensitivity to low concentrations. The log dose-response curve rose abruptly, at about 10^{-10} M, parallel to that of 5-hydroxytryptamine on the same heart (Fig. 6c). In both experiments, the equiactive molar ratio in this parallel portion of the curve was 0.1.

Yohimbine.—This alkaloid excites the *Venus* heart weakly. The threshold concentration is between 10^{-6} M and 10^{-5} M. The mean equipotent molar ratio is 630. The response is slow, taking about 30 min. for completion. It is also not entirely reversible. Evidence of specificity from tachyphylaxis experiments is negative but inconclusive.

DISCUSSION

The simplest structural requirement for 5-hydroxytryptamine-like activity on the *Venus* heart preparation seems to be embodied in phenethylamine and tryptamine: a flat aromatic nucleus with a 2-aminoethyl side-chain. These requirements are established largely by the ineffectiveness of some of the substances tested.

Neither the primary alkylamines, nor indole alone, have a 5-hydroxytryptamine-like action on the *Venus* heart. The n-alkylamines have been shown to be excitatory but unspecific in their action relative to 5-hydroxytryptamine (Greenberg, 1960). Evidently an alkyl side-chain cannot take the place of a proper ring substituent. Indole and its close congener skatole are likewise excitatory but unspecific in action. Indeed, these two compounds have been shown to be inactive

(Erspamer, 1952), or unspecifically depressant (Izquierdo and Stoppani, 1953), on a variety of smooth muscle preparations.

The characteristically different actions of histamine and dopamine on *Venus* heart (Greenberg, 1960) point to the ineffectiveness of imidazolyl- or dihydroxyphenyl-ethylamines in producing 5-hydroxytryptamine-like effects. However, if these compounds had such an action, it would probably be no stronger than that of tyramine. Hence, it might be completely masked by the strong generic effects of histamine and dopamine.

The importance of the amino-group itself is illustrated, firstly, by the ineffectiveness of indol-3-ylacetic acid, in which the amino-group is absent.

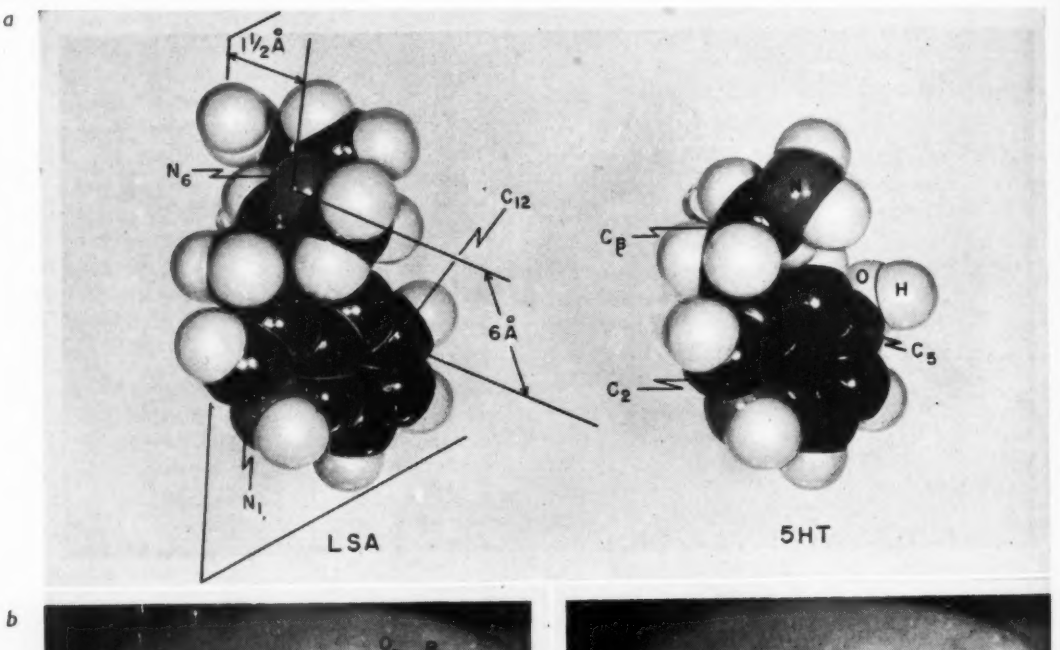


FIG. 7.—(a) Taylor-Hirschfelder models of (+)-lysergic acid (LSA) and 5-hydroxytryptamine (5HT). (b) Composite negative imprint of the molecules in (a) as a model of 5-hydroxytryptamine receptor area. Structural formulae of (+)-lysergic acid diethylamide (left) and 5-hydroxytryptamine (right) show orientation of molecules. The H at C_5 of (+)-lysergic acid diethylamide is omitted; R is $-N(C_2H_5)_2$. Three proposed negative binding sites for the 5-hydroxyl group, 1-nitrogen atom and terminal amino-group of 5-hydroxytryptamine are indicated.

Secondly, in tryptophan and 5-hydroxytryptophan solutions, the α-amino-groups are hydrogen-bonded to the carboxyl groups. These two tryptamine analogues are also feeble exciter agents on the *Venus* heart. Finally, the amino-nitrogen atom must be two carbon atoms distant from the ring. Pronounced reduction in potency accompanies either a decrease (gramine) or an increase [3-3-(dimethylaminopropyl)indole] of only about 1.5 Å. in the length of the aminoalkyl side-chain. That gramine excitation might not even be specific is suggested by the fact that it antagonizes the effect of adrenaline as well as 5-hydroxytryptamine in *Spisula solida* (Gaddum and Paasonen, 1955).

The arrangement in space of the aminoethyl side-chain of tryptamine and phenethylamine analogues is variable. Consequently, the effective conformations of these molecules and the corresponding shapes of the receptors are in question. A partial answer resides in two properties of (+)-lysergic acid diethylamide: Firstly, its extremely low threshold on the *Venus* heart suggests that the molecule must fit some cellular receptor site precisely. Secondly, the dimensions and conformation of the (+)-lysergic acid nucleus are fixed by closure of the C and D rings. Thus, lysergic acid diethylamide might well be used as a tool in establishing at least one active conformation and complementary receptor of tryptamine and its congeners.

Three salient points define the spatial arrangement of lysergic acid diethylamide (Cookson, 1953): (1) The lysergic acid nucleus is planar up to a line joining C_5 and C_8 across the upper (D) ring. (2) Along this line the D ring is folded so that N_6 (corresponding to the primary amino-nitrogen atom of 5-hydroxytryptamine) is out of the plane by 1 to 1.5 Å. (3) The distance between C_{12} (corresponding to C_5 in 5-hydroxytryptamine) and N_6 is between 5.5 and 6 Å.

Of the many possible conformations of 5-hydroxytryptamine, one exists (Fig. 7) which satisfies the spatial requirements defined by lysergic acid. (1) Only one of the three most probable positions of C_β, that in which the C_β hydrogen atoms are symmetrically disposed about the C_2 hydrogen atom, permits the carbon atoms of the aminoethyl side-chain of 5-hydroxytryptamine to lie in the same plane as the indole ring. Such a conformation is energetically favoured by the positioning of the C_2 and C_β hydrogen atoms at an energy minimum which, however, is not necessarily the lowest minimum (Dauben and Pitzer, 1956). (2) With C_β in the position described, the primary amino-nitrogen atom can be displaced 1 to 1.5 Å. out of the plane of the molecule by the rotation of C_α. (3) The distance between C_5 and the primary amino-nitrogen atom then varies between 4.5 and 6.5 Å. Thus, a receptor (for example, Fig. 7) which accommodates lysergic acid diethylamide will also accept 5-hydroxytryptamine—but only when the latter molecule is in the conformation described above. This conformation, it must be mentioned, is not the most probable one which exists for (2-aminoethyl)indole analogues. However, if the free energy barrier between this conformation and the most probable one is between 4,000 and 6,500 cal./mole, then for every 10^3 to 10^5 molecules having the probable conformation there will be one in the planar conformation hypothesized. Now, lysergic acid diethylamide requires about 10^6 to 10^7 times fewer molecules to produce a threshold response than do its tryptamine congeners. If we assume (comparing Table I and Fig. 6c) that lysergic acid diethylamide is between 10 and 100 times more potent than these analogues, then 1 out of 10^3 to 10^5 tryptamine molecules would indeed be sufficient to produce the observed effects. Thus, the improbability of the proposed conformation would seem to explain, in part, the great difference in the number of molecules required for the responses of lysergic acid diethylamide and its tryptamine analogues.

Two regions are seen to be necessary in the receptor area. Firstly, there must be a flat surface, about 11 Å. by 9 Å., which is complementary to the indole, or benzene, ring. Secondly, a contiguous ovoid depression, 6 Å. by 4 Å. and up to 3.5 Å. deep, should be present. The depression accepts the groups in the lysergic acid D ring and the terminal amino-groups of tryptamines or phenethylamines which are folded out of the plane of the indole ring.

On the basis of this model some deductions can be made regarding the binding forces between the various compounds employed and the receptor. The forces which bind the aromatic nuclei to the receptor are probably of the weak, unspecific van der Waals type which depend for strength on exact conformity between many atoms in the drug and its receptor. Also, the possibility of hydrogen bonding between the 1-nitrogen of the indole ring and the receptor exists. The relative impotency of the benzene nucleus in phenethylamine (compared to indole in tryptamine) could be attributed to a deficiency in either of these sorts of binding forces.

Since the pK_a of 5-hydroxytryptamine is 10.0, the amino-group is mostly ionized at physiological *p*H (Vane, 1959). This fact, together with the

loss of potency with lengthening of the side-chain (and presumably with distance from a binding site), suggests that the terminal amino-group of tryptamines, and probably of phenethylamines, is hydrogen bonded to some negative site in the receptor.

Structural modifications of the basic compounds, tryptamine and phenethylamine, affect both the potency and mode of action of the resulting analogues. The presence of the 5-hydroxyl group in 5-hydroxytryptamine, bufotenine and 5 - hydroxy - α - methyltryptamine renders these analogues more potent, faster acting, and more rapidly reversible than their unhydroxylated congeners. The potency relationship has been observed in other molluscan preparations. Gaddum and Paasonen (1955) found that tryptamine was about one thousand times less effective than 5-hydroxytryptamine in stimulating the heart of *Spisula solida*. The threshold of both 5-hydroxytryptamine and bufotenine excitation of *Anodonta* heart is 7×10^{-9} g./ml., while that of *N'N'*-dimethyltryptamine is found to be only 10^{-5} g./ml. (Marczyński, 1959). These data suggest the possibility of a hydrogen bridge between a negative site in the receptor (Fig. 7) and the 5-hydroxyl group which is unionized at physiological hydrogen-ion concentrations (Vane, 1959).

While tyramine is more potent and has a smaller response time than phenethylamine, it cannot be assumed that the *p*-hydroxyl group of tyramine necessarily occupies the same binding site as the 5-hydroxyl group of 5-hydroxytryptamine. A position of the molecule in which the benzene ring is flat on the planar portion of the receptor and the amino-group is in its appropriate site is compatible with binding of the *p*-hydroxyl group of tyramine either at the 5-hydroxyl site or the 1-nitrogen site of 5-hydroxytryptamine. The conformation of tyramine in either position is relatively improbable, which helps account for its low potency. Incidentally, if binding of the *p*-hydroxyl group at the 5-hydroxyl site occurs, an additional planar receptor area must be hypothesized. Both receptor positions, as well as others, are probably assumed by these small, flexible molecules.

Tryptamine analogues which have alkyl substituents on the primary amino-nitrogen atom have a lower potency than 5-hydroxytryptamine. However, in no case is potency significantly lower than that of tryptamine; in one instance (*N'*-methyltryptamine) it is higher. In this regard, an *N'*-methyl substituent, homologous with that of lysergic acid, would be expected to provide the most complementary fit to the hypothesized receptor.

Since the *N'*-alkyltryptamine effects are also irreversible, it becomes clear that increased binding alone seems to lead to little or no increase in potency if there is, at the same time, no 5-hydroxyl group. The hydrogen bridge at the latter location is essential for the precise positioning of the indole ring in the receptor area. If this is correct, then *N'*-alkyltryptamine analogues which have a 5-hydroxyl group should have a greater potency than 5-hydroxytryptamine. In fact, bufotenine is about thirty-five times as potent as 5-hydroxytryptamine, while 5-hydroxy-*N'*-methyltryptamine is about ten times as potent (Bumpus and Page, 1955). On the other hand, bufotenidine (5-hydroxy - *N'N'N'* - trimethyltryptamine) is only about as active as tryptamine on *Venus* heart (Twarog and Page, 1953). The amino-nitrogen atom in bufotenidine is quaternary. Consequently, it probably also acts on the acetylcholine receptors of the ventricle which would result in an observed effect with a negative inotropic component (Welsh and Taub, 1948).

Substitution of a methyl group in the tryptamine molecule, in positions other than on the primary amino-group, either lowers, or has little effect on, the potency of the resulting compound. Although such compounds are also irreversible indicating strong binding, the analogues with 5-hydroxyl groups, unlike the *N'*-alkyl tryptamines, are not much more potent than 5-hydroxytryptamine. Thus, 5-hydroxy-2-methyltryptamine is barely more potent than 5-methoxy-2-methyltryptamine. Again, while the presence of a 5-hydroxyl group in α-methyltryptamine changes the character of the response, the potency remains essentially unchanged. Substitution of a methyl group in positions other than on the primary amino-group probably sterically hinders the binding of the side-chain (α-methyltryptamines) or the indole nucleus (2-methyltryptamines) by lifting them off the planar portion of the receptor. For example, a methyl group or bromine atom in the 2-position would raise C_3 and the adjacent tertiary nitrogen atom almost an Ångstrom, thus, of course, decreasing the strength of the interaction. If the displacement is sufficient, the presence or absence of the 5-hydroxyl group seems to have relatively little further effect on the positioning, and hence the potency, of the analogue.

Conformations of 5-hydroxytryptamine other than that represented by the hypothetical receptor exist, of course. Some of these might also be

effective on the *Venus* heart. For example, another spatial arrangement is represented in the structure of the harmala and rauwolfia alkaloids. As is well known, both 5-hydroxytryptamine-like action and inhibition of 5-hydroxytryptamine-like action by these compounds have often been correlated with the presence of the 3-(2-aminoethyl)-indole configuration (Woolley and Shaw, 1957). However, two features of yohimbine, in particular, suggest that such a conformation of 5-hydroxytryptamine is ineffective on *Venus* heart.

First, it is obvious that when the planar indole rings of lysergic acid diethylamide and yohimbine are congruent, the important ring nitrogen atoms (N_6 and N_4 respectively) are 4 to 5 Å apart. Thus, judging from the lack of potency of gramine and 3-(3-dimethylaminopropyl)indole, yohimbine and similar alkaloids cannot be effective at the *Venus* heart receptors with which lysergic acid diethylamide interacts. It follows that 5-hydroxytryptamine in a conformation corresponding to that of yohimbine (and involving, at least, rotation of C_β through about 90°) would also be inactive at the lysergic-acid-like site. Second, not only is the structure of yohimbine theoretically incompatible with effective action at lysergic-acid-like sites but also it is, *in fact*, relatively inactive. This suggests that, in *Venus* heart, there is no receptor area complementary to yohimbine or to 5-hydroxytryptamine in this conformation.

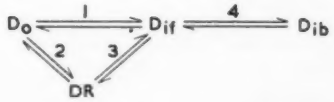
It seems possible that the well-known differences in action between the rauwolfia and ergot alkaloids (Brodie, 1958) are due to the fact that these two groups of compounds are structurally incapable of acting at the same cellular receptors. The relative smallness and flexibility of 5-hydroxytryptamine, on the other hand, would permit interaction of this molecule with the sites of both sorts of alkaloids. However, differences between such interactions would be expected and, in fact, occur in a variety of preparations (Woolley and Shaw, 1953; Gaddum and Hameed, 1954; Salmoiraghi and Page, 1957).

Tissues may have a population of cellular receptors representing the different conformations (and presumably the different effects) of small, active molecules such as 5-hydroxytryptamine. The relative effectiveness of large molecules with fixed conformations would be a measure of the distribution of such a population. The *Venus* heart receptors, in contrast to those of mammalian brain, apparently respond to a smaller range of possible conformations of 5-hydroxytryptamine.

Many analogues of tryptamine are more or less irreversible. The ease with which a drug will leave the tissue for the bath solution is determined, in part, by the oil-water partition coefficient of the compound. Vane (1959) showed that the coefficient of tryptamine is about twenty times that of 5-hydroxytryptamine due to the absence, in the former, of the polar hydroxyl group. It is well known that alkyl substitution increases the partition coefficient and it is in these terms that the irreversibility of such compounds can most readily be explained.

There is no evidence from experiments on the *Venus* heart preparation which indicates whether the *N'*-alkyltryptamines are merely strongly bound to the cell membrane or whether they actually penetrate. However, data from other studies suggest that these compounds enter the cell. Vane (1959), for example, using the enhancement of the excitor activity by amine oxidase inhibition as a test, was able to show that tryptamine analogues with high oil-water partition coefficients (for example, tryptamine, *N'*-ethyltryptamine, *N'*-methyltryptamine) entered the cells of the rat fundus while 5-hydroxytryptamine did not. Similarly, lysergic acid diethylamide and bufotenine, as well as *N'N'*-dimethyltryptamine and *N'N'*-diethyltryptamine (Szara, 1957), are hallucinogens in man and thus cross the blood-brain barrier. 5-Hydroxytryptamine, which cannot cross, is not a hallucinogen.

If the alkyl-substituted tryptamines enter the cell, then the effective drug molecules must be considered as being divided into two pools: those inside (D_i) and those outside (D_o) the cell. The following equilibria might obtain at any bath concentration:



where D_{if} and D_{ib} are, respectively, free and bound drug inside the cell. DR is drug-receptor complex at the cell surface. The following points need to be made regarding the above model:

(1) The action of lysergic acid diethylamide and the *N'*-alkyltryptamines suggests that the drug accumulates slowly in the cells from low bath concentrations. However, it is impossible, on present evidence, to distinguish between steps (1) and (2+3). It is important to note that the response is assumed to be a measure of the concentration of drug-receptor complex. Consequently, even if step (1) does occur, only internal

free drug accumulating in this manner in close proximity to the receptor can be measured.

(2) The biphasic dose-response curves which are obtained in a third of *N'*-alkyltryptamine experiments (Fig. 4c) suggest that the "tryptamine space" of the cell can be saturated. Thus, when the bath concentration is about 10^{-8} M the net movement of drug into the cells ceases. The ratio of dose-response becomes constant until the bath concentration is made large enough (about 10^{-7} M) so that $d(D_oR)/dt$ becomes larger than $d(D_iR)/dt$. Naturally, compounds such as the 2-methyl analogues, whose thresholds are greater than the maximum internal drug concentration, will have no effect even though they enter the cell and accumulate there to the same extent as do the *N'*-alkyltryptamines.

(3) If drug is bound in the cell, it is not bound to the receptors (that is, $D_{ib} \neq DR$). As drug concentration is increased above the levels which saturate the cell interior, the response again increases, indicating the availability of more free receptors. Lipid associated with the cell membrane is probably responsible for intracellular binding if the latter occurs.

The relationship between doses of lipid-soluble tryptamine analogues and their effects on the *Venus* heart is obviously a complex one. The individual variation found in the responses of different hearts to the *N'*-alkyltryptamines clearly reflects this.

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THE EFFECTS OF ADRENALINE AND NORADRENALINE ON THE METABOLISM AND PERFORMANCE OF THE ISOLATED DOG HEART

BY

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In 16 dog heart-lung preparations modified to permit a more accurate measurement of coronary flow, adrenaline or noradrenaline was infused at a rate of 4 μg. base/min. After a 30-min. pause during which the increased oxygen consumption and heart rate, but not the coronary flow, returned to pre-infusion levels, the other sympathomimetic amine was infused for the same length of time. It was found that, mole per mole, noradrenaline is as effective, and probably more so, than adrenaline in raising the oxygen consumption of the heart-lung preparation. The positive chronotropic and coronary dilating action of both amines appear to be equal. It was observed that in any one experiment the second dose of the sympathomimetic amine was slightly more effective than the first dose in raising the oxygen consumption. The level of high-energy phosphorus compounds does not change after adrenaline or noradrenaline administration even at the time when the oxygen consumption rises to as much as 200%. During this period there are no signs of cardiac hypoxia, as can be judged by the good oxygen saturation of coronary venous blood. Single doses of 5 μg. adrenaline or noradrenaline have a consistent positive inotropic effect that lasts about 15 min. when tested on a failing heart. In 12 experiments on non-failing modified heart-lung preparations, a single dose of 5 μg. adrenaline fails to cause a measurable increase in oxygen consumption after 1, 3, 6, or 11 min. in spite of a mild positive chronotropic action. The significance of these findings is discussed and the suggestion made that, when noradrenaline infusions are effective in treating cardiogenic shock in man, part of this effect may be due to its positive inotropic action, thus correcting an element of heart failure that might exist.

The main actions of adrenaline on the heart are: positive inotropic and chronotropic effects, increased atrio-ventricular conduction, and increased excitability. In addition to these, there is the "calorigenic" effect. This is the increase in cardiac oxygen consumption which cannot be explained solely by the rise in heart rate and increase in work performed (for literature see Gollwitzer-Meier, Kramer and Krüger, 1936). All these effects of adrenaline need not have the same mechanism of action at the cellular or subcellular (enzymatic) level. Krayer (1949), for instance, has shown that the chronotropic action but not the inotropic action can be inhibited by veratramine. It is also conceivable that a certain dose of adrenaline would evoke one effect and not the other. The mechanism of the coronary dilating action of adrenaline is not completely understood and it is not clear to what extent the vasodilatation is due to the rise in cardiac metabolism.

In this study a comparison has been made between the "calorigenic," chronotropic, and coronary dilating actions of (−)-adrenaline and (−)-noradrenaline on the dog heart-lung preparation and the effect of these compounds on the phosphorus compounds of the heart. Furthermore, a study has been made of the effect of graded doses of adrenaline, to see if the inotropic action can be separated from the calorigenic action. This study appeared important in view of statements often encountered in the clinical literature that (*a*) adrenaline raises the blood pressure mainly by virtue of its action on the heart, increasing the cardiac output, whereas noradrenaline acts peripherally; (*b*) adrenaline is contraindicated in the treatment of cardiogenic shock as it increases myocardial oxygen consumption and produces symptoms of coronary

TABLE 1

EFFECT OF ADRENALINE AND NORADRENALINE ON THE ISOLATED DOG HEART

Adrenaline added to reservoir at rate of 4 μg./min. for 15 min. followed by a 30 min. pause, then a 15 min. infusion period with noradrenaline. The next day the order was reversed, noradrenaline added first. The values at the end of the second 15 min. period are compared with those at 45 min. as a base line. The numeral (1) indicates at the start of the experiment, (2) after 15 min., (3) after 45 min., (4) after 60 min.

	Part 1								Part 2							
	Adrenaline				Noradrenaline				Adrenaline				Noradrenaline			
No. of expts. ..	16				16				8				8			
	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
Heart rate	138	—	141	—	—	—	—	—	—	—	—	—	—	—	—	—
Mean±S.E. ..	(±4·8)		(±2·3)													
% change in heart rate	—	44·3	—	44·3	—	47·1	—	47·1	—	47·8	—	41·0	—	57·0	—	37·3
Mean±S.E. ..		(±3)		(±3)		(±4·5)		(±4·5)		(±4·6)		(±3·6)		(±5·9)		(±5·0)
Oxygen consumption (ml./100 g./min.) ..	6.67	—	—	—	6.67	—	—	—	—	—	—	—	—	—	—	—
Mean±S.E. ..	(±0·38)				(±0·38)											
% change in oxygen consumption ..	—	74·0	−3·7	74·0	—	112·0	−3·7	112·0	—	54·2	—	93·8	—	98·9	—	124·9
Mean±S.E. ..		(±11·6)	(±3·3)	(±11·6)		(±16·0)	(±3·3)	(±16·0)		(±9·9)		(±19·3)		(±27·5)		(±16·6)
Mean % change in work performed ..	—	−1·37	−8·1	−1·37	—	−0·8	−8·1	0·8	—	−2·35	—	−0·4	—	+2·2	—	−3·9
Coronary flow (ml./min.)	55	121	151	215	55	121	151	215	—	—	—	—	—	—	—	—
Mean±S.E. ..	(±6)		(±26)		(±6)		(±26)									
% change in coronary flow	—	—	—	—	—	—	—	—	—	113·6	—	78·9	—	130·7	—	49·4
Mean±S.E. ..										(±12)		(±20·8)		(±26·1)		(±20)

Phosphorus Compounds of Heart Muscle. Mean±S.E. (% Total Acid-soluble Phosphorus)	
Phosphocreatinine phosphorus	25·3 (±0·94)
Inorganic phosphorus	17·8 (±0·87)
Labile nucleotide phosphorus (2/3 of adenosine triphosphate)	27·7 (±0·62)

Labile nucleotide phosphorus (2/3 of adenosine triphosphate)	27·7 (±0·62)

TABLE II

EFFECT OF 5 μG. ADRENALINE ON THE ISOLATED DOG HEART

In the first five experiments adrenaline was added to the reservoir. In the next seven experiments adrenaline was injected into the venous cannula near the right heart, and in this series the first oxygen consumption measurement was made 45 sec. (4 experiments) and 1 min. after the injection; since the readings at 45 sec. and 1 min. were not different they were averaged together. Initial coronary flow was 56 (±6·6) in the experiments in which adrenaline was added to the reservoir, and 56 (±4·8) in those in which adrenaline was injected.

	Adrenaline Added to Reservoir						Adrenaline Injected					
No. of expts.	5						7					
Min. after injection	1	3	6	8	11	13	1	3	6	8	11	13
% change in heart rate	+13·5	+19	+15	+11·7	—	+11·0	27·0	+11·2	+10·4	—	+6·1	—
Mean±S.E.		(±2)				(±1·3)	(±5·9)		(±3·6)		(±2·4)	
% change in oxygen consumption ..	—	+3·8	—	−3·1	—	−2·2	+3·8	—	+3·0	—	−5·9	—
Mean±S.E.		(±1·8)		(±2·5)		(±3·1)	(±3·6)		(±2·7)		(±2·2)	
Mean % change in work performed	—	+4·4	—	+3·9	—	+2·7	+0·6	—	+0·6	—	+1·2	—
% change in coronary flow	—	+23·4	—	+38·6	—	+41·7	+11·9	—	+35·2	—	+45·3	—
Mean±S.E.		(±6·1)		(±12·3)		(±11)	(±2·3)		(±9·2)		(±11·2)	
Phosphorus Compounds of Heart Muscle (% of Total Acid-soluble Phosphorus)												
Phosphocreatine phosphorus ..				25·1 (±2·0)						26·0 (±0·8)		
Inorganic phosphorus				12·6 (±1·03)						13·9 (±0·89)		
Labile nucleotide phosphorus (2/3 of adenosine triphosphate) ..				30·3 (±0·86)						29·2 (±1·21)		

insufficiency, whereas noradrenaline does not increase cardiac oxygen consumption.

METHODS

Male and female dogs were used weighing about 12 kg. The techniques of preparing the heart-lung, of measuring coronary flow and oxygen consumption, as well as other experimental details have been described previously (Fawaz and Tutunji, 1959). Blood pressure was maintained at 100 mm. Hg, and an attempt was made to keep the left ventricular output constant. The average left ventricular output in this series was about 650 ml./min. Readings were taken at the start of the experiment, and then one of the sympathomimetic amines was added to the venous reservoir at the rate of 4 μg./min. for a period of 15 min. at the end of which measurements were again taken. After a period of 30 min. without infusion readings were taken again and an equimolar amount of the other sympathomimetic amine added over a period of 15 min.; after this the final readings were taken. The next day the order of adding the sympathomimetic amines was reversed. In all, 16 such experiments were performed over a period of a whole year. Each sympathomimetic amine was given 8 times during the first 15 min. and 8 times during the last 15 min. of the experiment. Experiments were terminated at the end of the second 15-min. infusion period and a piece of the left ventricle was excised for the determination of the phosphorus compounds.

The inotropic action of adrenaline and noradrenaline was tested on the classical Starling heart-lung preparation. Failure was produced in the course of about 15 min. by repeated additions of pentobarbitone until the systemic output fell to about half the original level with a corresponding rise in right auricular pressure. A single dose of 5 μg. of adrenaline or equimolar amount of noradrenaline was added to the reservoir or injected into the venous inflow tube near the right heart. The calorigenic action of this dose of adrenaline was tested on the modified non-failing heart-lung preparation by injection into the venous inflow tube, and the usual measurements were taken 45 sec., 1, 3, 6, 11, and 13 min. thereafter. The procedure for measuring oxygen consumption in these "acute" as well as in the infusion experiments was as follows: Coronary flow was measured during an interval of 15 sec., coronary venous and arterial blood samples were then collected simultaneously for the oxygen determination by the method of Van Slyke, a procedure that requires 5 sec., then a second coronary flow measurement was made, and the average was taken of the two coronary flow figures, which usually agreed very well.

RESULTS

Comparison of the Calorigenic, Chronotropic, and Coronary-dilator Actions of Adrenaline and Noradrenaline

It can be seen from Table I that during the 30-min. pause following the first 15-min. infusion period both the heart rate and the oxygen consumption return to pre-infusion levels. Yet we took not only the average of all 16 measurements for each of the sympathomimetic amines (part 1 of Table I), but made separate analysis of the 8 experiments during which each amine was given at the beginning or at the end (part 2 of Table I). We noticed early that the same amine, given at the end of the experiment, tends to have a greater calorigenic action and the same or slightly smaller chronotropic action than when given at the beginning of the experiment. We have no explanation for this finding, which may or may not be significant. Another reason for this separate analysis is because the coronary flow does not return to normal and may even decrease during the 30-min. pause.

"Calorigenic Action."—It is quite clear from the figures presented in Table I that one can speak of a "calorigenic" action of noradrenaline and adrenaline, for, in the absence of a change in work performed, a tachycardia with a 44 to 47% increase in rate cannot explain this rise in oxygen consumption. In a previous publication (Fawaz and Tutunji, 1959) we have shown that a tachycardia with about 65% increase in rate can result in not more than 23% increase in oxygen consumption. Our results indicate that the cardiac calorigenic action of noradrenaline is at least equal to, and probably greater than, that of adrenaline. There are marked variations in the response of different dogs to adrenaline and noradrenaline. The initial cardiac oxygen consumption, however, does not vary so much from one animal to another and in this particular series of 16 hearts it is 6.67 ± 0.38 ml./100 g./min. Due to these variations in response and the resulting high standard error it is not possible to compare the calorigenic actions of adrenaline and noradrenaline in a strictly quantitative manner. Gollwitzer-Meier, Kramer and Krüger (1936) and Gollwitzer-Meier and Witzleb (1952) have had the same experience regarding the variations in response to single doses of adrenaline and noradrenaline.

Chronotropic Action.—Our results indicate that adrenaline and noradrenaline infused at the rate of 4 μg./min. are equally effective in increasing the heart rate in the denervated heart-lung preparation. The heart rate rises rapidly and remains more or less steady during the infusion. Furthermore, we have found less variation from one animal to another in the chronotropic response to the sympathomimetic amines than in

the "calorigenic" response. Krayer (1949) and Krayer and Van Maanen (1949) have also found that the heart rate remains steady during a constant infusion of adrenaline or noradrenaline

Coronary Dilating Action.—Both adrenaline and noradrenaline given at the rate of 4 μg./min. have a definite coronary dilating action as can be seen from Table I. During the first 15 min. there was a gradual increase in coronary flow amounting, at the end of this period, to 113% in the case of adrenaline and 130% in the case of noradrenaline. It is known that coronary flow increases spontaneously in a heart-lung preparation, but the "spontaneous" rise in coronary flow during the first 15 min. is much less than that observed after the addition of sympathomimetic amines. This can be seen by comparing these values with results reported in a previous publication (Fawaz and Tutunji, 1959) and obtained under almost identical experimental conditions. These results, however, do not answer the question as to what extent the effect of the sympathomimetic amines is secondary to the marked increase in oxygen consumption.

The Inotropic Action of Adrenaline or Noradrenaline

If 5 μg. of adrenaline or noradrenaline is injected into the venous inflow tube near the right heart or into the venous reservoir in a classical Starling heart-lung preparation previously made to fail with pentobarbitone, the results are clear-cut and reproducible. There is a sudden drop of right auricular pressure almost to pre-failure level, then a gradual rise to pre-injection level in the course of about 15 min. Simultaneously there is an increase in output almost to pre-failure level followed by a gradual decrease to pre-injection level. The blood pressure rises suddenly to a high level during the first 15 sec. if the amine is injected near the heart and only slightly if injected into the reservoir. If the injection is near the heart the heart rate also increases by about 27% during the first minute and subsides to normal within 5 to 10 min. If now 5 μg. of the other sympathomimetic amine is injected the result is almost identical except that the correction of failure is not as complete. Adrenaline and noradrenaline behave identically in this respect. This experiment has been performed many times and is a student demonstration experiment. An illustration of this action can be seen in figure 12 of Krayer (1949) demonstrating the positive inotropic effect of 10 μg. of adrenaline in a failing heart-lung preparation treated with veratramine.

The Lack of Measurable Calorigenic Effect of 5 μg. of Adrenaline

The calorigenic effect of 5 μg. adrenaline was tested on the non-failing heart-lung preparation performed according to the modified procedure to allow a more accurate measurement of coronary flow (Table II). In 7 experiments, 5 μg. adrenaline produced no measurable increase in oxygen-consumption 45 sec., 1, 6 or 11 min. after injection. In another 5 experiments where 5 μg. adrenaline was added to the reservoir, a procedure known to produce definite inotropic effect, again no increase in oxygen consumption occurred after 3, 8, or 13 min.

The heart rate did increase in these experiments, by a maximum of about 27% in the first series, but this increase was not accompanied by a measurable increase in oxygen consumption.

The Effect on the High-energy Phosphorus Compounds of Heart Muscle

As can be seen from the tables, there was no change in the high-energy phosphorus compounds of heart muscle even in those experiments where the oxygen consumption rose to as much as 200%. During this period, however, the coronary flow was increased and there were no signs of cardiac hypoxia as can be judged by the high oxygen content of coronary venous blood.

Discussion

The results indicate that infusion of noradrenaline is as effective as adrenaline in raising the oxygen consumption of the isolated denervated dog heart. A quantitative evaluation of the actions of the two amines is not possible owing to the high standard errors resulting from the great individual variations in the response to the sympathomimetic amines from dog to dog. Gollwitzer-Meier and Witzleb (1952) are the only investigators who have studied the calorigenic effect of noradrenaline on the isolated denervated dog heart and they came to the opposite conclusion. They injected single doses of noradrenaline (20 to 40 μg.) and adrenaline (10 to 20 μg.). The maximum increase in oxygen consumption after noradrenaline was 21 to 154% (15 experiments) and 54 to 252% after adrenaline (6 experiments). They thus concluded that the metabolic action of noradrenaline was substantially less ("wesentlich geringer") than that of adrenaline. The experimental part of Gollwitzer-Meier and Witzleb's work was different from ours. In the first place, these authors used single injections of adrenaline and noradrenaline and noted the peak in oxygen

consumption. We, however, infused these substances at a constant rate of 4 μg./min. for 15 min. and measured the oxygen consumption at the end of this period. We have not measured the oxygen consumption during this 15-min. period. However, we have measured the heart rate during this interval and found it to be relatively constant, thus confirming the observations of Krayer (1949) and Krayer and Van Maanen (1949) with adrenaline and noradrenaline. There are also other differences in the experimental conditions of Gollwitzer-Meier and those of the present experiments. For example, in the methods for measuring coronary flow and oxygen content of the blood.

Our results also indicate that noradrenaline is at least as effective as adrenaline in increasing the heart rate and also the coronary flow. Whether or not the increase in coronary flow is secondary to the increased myocardial oxygen consumption cannot be decided from our experiments, which were not originally planned to answer this specific question. Even in experiments where single doses of 5 μg. adrenaline were given and found to be without measurable effect on oxygen consumption, the rise in coronary flow could not be distinguished from the "spontaneous" increase observed in heart-lung preparations. It has been known for some time that noradrenaline can cause coronary vasodilatation (see Lochner, Mercker and Schürmeyer, 1956), but no quantitative study has been made of its efficacy in comparison with adrenaline.

As was stated above, we found that single injections of 5 μg. adrenaline had no measurable effect on the oxygen consumption of the denervated heart-lung preparation. Here we find ourselves in complete disagreement with Gollwitzer-Meier *et al.* (1936), who state that doses of 5 to 10 μg. adrenaline increase the oxygen consumption of the denervated heart-lung preparation 100 to 250% and even 350%, the effect reaching a maximum 1 min. after injection and lasting 5 to 10 min. We have not found a measurable increase in oxygen consumption 45 sec., 1, 3, or 6 min. after injection, even when we injected the adrenaline into the venous cannula near the right heart as Gollwitzer-Meier *et al.* did. We believe that differences in experimental technique may account for this discrepancy in results. In the first place, the methods of measuring the coronary flow and thus the technique of preparing the heart-lung are different. Gollwitzer-Meier *et al.* employed a Morawitz cannula to measure coronary sinus flow and calculated from this the total coronary flow. In a previous publication (Fawaz, Hawa and Tutunji, 1957), we have given our reasons why we believe that the Morawitz cannula method cannot be relied upon to measure even total coronary sinus flow. Although both groups utilize the Fick principle for measuring oxygen consumption, we measure coronary flow and blood oxygen directly whereas Gollwitzer-Meier *et al.* use indirect methods for measuring coronary sinus flow (Weese's Stromuhr and Rein's Thermostromuhr) and blood oxygen content (Kramer's photoelectric method). We are not in a position to judge these indirect methods; however, all indirect methods have eventually to be calibrated by direct measurements with a graduated cylinder or a Van Slyke apparatus. The indirect methods have the general advantage of yielding continuous and instantaneous measurements. In this particular case there is no advantage in that, for knowledge of the oxygen content of coronary sinus blood at any one instant obviously does not tell us about the metabolic activity of the cardiac cell at that particular instant.

A dose of 5 μg. adrenaline did cause a measurable increase in heart rate, about 27%, after 45 to 60 sec., and theoretically an increased oxygen consumption is to be expected in view of the known effect of tachycardia *per se* in increasing metabolism (Laurent, Bolene-Williams, Williams and Katz, 1956; Berglund, Borst, Duff and Schreiner, 1958; and Fawaz and Tutunji, 1959). However, Laurent *et al.* found in the intact animal an 11% increase in oxygen consumption with a 27% increase in heart rate and a 5.5% increase in oxygen consumption with a 36% increase in heart rate. Badeer and Khachadourian (1958) observed no significant reduction in oxygen consumption when the heart rate was reduced from 153 to 110 beats per min. by applying a cold thermode to the sino-atrial node. Clearly, such changes in oxygen consumption are within the limits of experimental error.

The observation that small doses of adrenaline can have a positive inotropic action but no measurable calorigenic action may be of considerable interest. Noradrenaline has been used in recent years in the treatment of shock due to myocardial infarction (Binder, Ryan, Marcus, Mugler, Strange, and Agress, 1955), with the belief that noradrenaline acts peripherally to raise blood pressure whereas adrenaline acts mainly in the heart. The observation that adrenaline injections may sometimes produce symptoms of coronary insufficiency helped to support this hypothesis.

Perhaps if the clinicians had known that noradrenaline could raise the myocardial oxygen consumption to the same extent as adrenaline they would have hesitated to use it in cases of shock due to myocardial infarction. However, it is quite possible that, where noradrenaline is of use in cardiogenic shock, its efficacy may be due precisely to its cardiac (inotropic) action. The element of cardiac failure in shock—even haemorrhagic shock—has hitherto received little attention (for review of literature see Walton, Richardson, Walton and Thompson, 1959) and it may well be that the doses of noradrenaline used clinically have no effect on raising the cardiac oxygen consumption but produce a positive inotropic action, thus counteracting any existing element of cardiac failure. Noradrenaline is to be preferred to adrenaline in this treatment because the latter, by raising the basal metabolic rate—a property not shared by noradrenaline—indirectly puts an extra burden on the heart. The increase in cardiac output observed after adrenaline may thus be due to such a general metabolic action and be similar to the action of the thyroid hormones.

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THE ANTI-ANAPHYLACTIC AND HISTAMINE-RELEASING PROPERTIES OF THE ANTIHISTAMINES. THEIR EFFECT ON THE MAST CELLS

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It has been shown that, depending upon their concentration, antihistamines act in three different ways: (*a*) by competitive inhibition of histamine as already known; (*b*) by destroying mast cells and releasing histamine; and (*c*) by preventing mast cell damage and histamine release in anaphylaxis. Furthermore, antihistamines potentiated mast cell damage and histamine release by compound 48/80, when acting on guinea-pig tissues, and inhibited these same phenomena when acting on rat tissues. It is concluded that the effect of antihistamines in anaphylaxis is possibly due both to their competitive inhibition of histamine on smooth muscle receptors and to their inhibition of mast cell damage and histamine release by antigen.

It is known that the release of histamine in anaphylaxis is accompanied by damage to the mast cells (Mota, 1953; 1958; 1959; Mota and Vugman, 1956; Humphrey and Mota, 1959). It has been suggested that antihistamines may act either by blocking tissue receptors to circulating histamine or by preventing the release of histamine from the tissues (Dale, 1950). If the latter is true, antihistamines should protect mast cells from damage induced by anaphylaxis. Our experiments have shown that antihistamines can act not only as inhibitors of mast cell damage and histamine release in anaphylaxis but also as very potent histamine releasers.

METHODS

Wistar rats and guinea-pigs of either sex, and of body weight 200 to 400 g., were used in all experiments.

Sensitization.—Rats were sensitized by injecting intraperitoneally 1 ml. of horse serum plus 1 ml. *Haemophilus pertussis* vaccine containing 20,000 million organisms. The rats were used from 2 weeks after sensitization. Evidence of sensitization was provided by testing whether the mast cells presented extrusion of the granules in presence of the specific antigen; a tiny piece of mesentery was first incubated with antigen and afterwards fixed and stained for mast cell observation. Animals whose mesentery did not present mast cell disruption on contact with antigen were discarded. Guinea-pigs were passively sensitized by injecting intravenously 0.5 ml. of rabbit antiserum containing 1.2 mg. antibodies to ovalbumin and used 12 to 72 hr. after the injection of antiserum.

Antihistamines.—The following antihistamine drugs were used: antazoline hydrochloride (Antistine, Ciba), chlorpheniramine maleate (Alergon, Schering, and Polaramine, Schering), diphenhydramine hydrochloride (Benadryl, Parke-Davis), bromodiphenhydramine hydrochloride (Ambodryl, Parke-Davis), isothipendyl hydrochloride (Andantol, Homburg), mepyramine hydrogen maleate (Eulantinon, Laborterápica; Anthisan, May and Baker), methapyrilene hydrochloride (Histadyl, Lilly), phenindamine hydrogen tartrate (Thephorin, Roche), promethazine hydrochloride (Phenergan, Rhodia), and tripelennamine hydrochloride (Pyribenzamine, Ciba). The authors are grateful to the drug firms mentioned for supplies of the pure crystalline salts. Advantage was taken of the observation that bromodiphenhydramine, diphenhydramine and mepyramine lost their antihistamine activity after boiling for 10 min. with 20% hydrochloric acid, and thus the experiments concerning histamine release were performed with these three antihistamines while the effect on the mast cells was studied with all of them.

Removal and Treatment of the Tissues.—The animals were killed by a blow on the head, bled from the jugular veins and their tissues removed immediately. The skin was first shaved with an electric c ipper and cut in pieces of about 1 sq. cm. The lung was cut in slices 0.8 mm. thick with the McIlwain tissue chopper, washed for about 2 hr. in a large vol. of cold Tyrode solution, and 4 or 5 of these slices were used as a sample. The diaphragm was divided into 2 symmetrical halves each being used as a sample. The stomach and ileum were first opened and their contents washed out with cold Tyrode solution. The mesentery was dissected away from the small intestine and cut into several small pieces, which were dipped into cold Tyrode solution, 2 or 3 of which were used as a sample.

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Experiments to Study Histamine Release and Mast Cell Damage.—To study the histamine-releasing property of the antihistamines, rat or guinea-pig tissue samples were shaken gently in 50 ml. beakers closed with parafilm caps for a standard time of 15 min. at 37° in 4 ml. of Tyrode solution containing the required concentration of antihistamine, after which the tissue samples were transferred to the hydrochloric acid solution for histamine extraction or to fixative for mast cell observation. As a control in each experiment a tissue sample of similar size and thickness was incubated in Tyrode solution with no added drug.

Experiments to Study Inhibition of Histamine Release and Mast Cell Damage in Anaphylaxis.—Tissue samples were first incubated for 15 min. at 37° in Tyrode solution or Tyrode solution containing the antihistamine, washed for 5 min. in Tyrode solution and then transferred to Tyrode solution containing antigen. In the experiments with guinea-pig tissues ovalbumin was added to give a final concentration of 1 mg./ml., and in the experiments with rat tissues horse serum was added to produce a 10% solution. The tissues were then incubated for a further period of 15 min. and transferred to the hydrochloric acid solution for histamine extraction or to fixative for mast cell observation. Histamine was assayed in all the incubation fluids. By means of these experiments the histamine release by antigen in non-treated control samples, the histamine release by the antihistamine itself, and the histamine release by antigen after previous contact of the tissue with the antihistamine could be determined.

Histamine Extraction and Assay.—After incubation all the tissues were cut into small pieces and transferred to 20% hydrochloric acid solution. Acid was also added to the incubation fluids to give a final concentration of 20% and then both the extracts and incubation fluids were boiled for 10 min. to destroy antihistamines and evaporate excess acid. Later the extracts and incubation fluids were neutralized with sodium hydroxide and diluted to the required volume with Tyrode solution. The extracts were assayed against histamine acid phosphate on the atropinized guinea-pig ileum. Histamine loss in the controls was subtracted from that released by antihistamines. Histamine release was expressed as a percentage of the total tissue histamine. In order to exclude any non-histamine component, the solutions were reassayed after antagonism of histamine by mepyramine 10^{-7}.

Mast Cell Observation.—The mesentery is a very suitable preparation for the study of mast cells. The description of mast cell damage presented here is based on observations in this tissue. Guinea-pig mesentery was fixed in a 4% solution of lead subacetate in 50% ethanol plus 1% acetic acid (Mota and Vugman, 1956) and stained in a 0.1% toluidine blue solution. Rat mesentery was fixed and stained in a 10% formaldehyde solution containing 1% toluidine blue and 0.1% acetic acid. The mesentery was examined as a whole-mount preparation. Rat mast cell damage was assessed by counting the percentage of cells presenting granules extrusion, 500 cells being counted. Guinea-pig mast cell damage was assessed by counting the total number of cells containing metachromatic granules in 30 microscopical fields with a magnification of 130.

Phase Contrast Observation.—In order to observe directly the effect of antihistamines on the mast cells, rat peritoneal mast cells were isolated by the method of Padawer and Gordon (1955) but using a 30% solution of bovine serum albumin as the high density medium, and the behaviour of these cells when contacting the antihistamines was observed by phase contrast microscopy. A drop of the isolated mast cell suspension was placed on a slide and covered with a square coverslip, two sides of which were sealed with melted paraffin. A small well of paraffin was then made at each free side of the coverslip in the same way as was described by Strangeways and Canti (1927), the preparation kept in a warm stage at 37°, and a suitable group of well-preserved mast cells selected for observation. A drop of 0.1% solution of diphenhydramine, mepyramine or bromodiphenhydramine was then placed in one free side of the coverslip, sucked on to the cells with a piece of filter paper on the other side of the coverslip, and the response of the cell observed with an oil immersion objective (1,000).

Results

Experiments with Guinea-pig Tissues

Histamine Release by Antihistamines from Guinea-pig Tissues.—Samples of guinea-pig skin, lung, diaphragm, stomach, ileum, and mesentery were incubated with 0.001 M bromodiphenhydramine, 0.001 M diphenhydramine, and 0.001 M mepyramine and the amount of histamine released determined. The results are shown in Table I. The antihistamines released considerable quantities of histamine

TABLE I

HISTAMINE RELEASE *IN VITRO* BY ANTIHISTAMINES FROM GUINEA-PIG TISSUES

Histamine release is expressed as a % of the total tissue histamine. Each figure represents the result of a different experiment.

Tissue	% Histamine Release by								
	Bromodiphenhydramine HCl 0·001 M			Diphenhydramine HCl 0·001 M			Mepyramine Acid Maleate 0·001 M		
Skin ..	10	12	15	9	10	13	6	9	10
Diaphragm	30	70	60	51	61	29	21	39	31
Stomach ..	0	0	2	0	0	5	2	2	0
Ileum ..	13	11	14	12	13	11	12	14	9
Mesentery	76	86	59	80	79	49	39	41	50
Lung ..	69	79	60	48	51	58	40	25	29

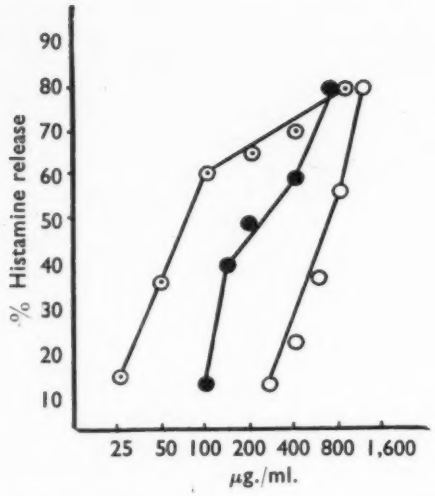

FIG. 1.—Effect of concentration of antihistamines, bromodiphenhydramine ⊙——⊙, diphenhydramine ●——●, and mepyramine O——O, on histamine release from guinea-pig lung. Ordinates: histamine release expressed as a % of the total tissue histamine. Abscissae concentration of antihistamine in μg./ml. Each point is mean of 2 experiments.

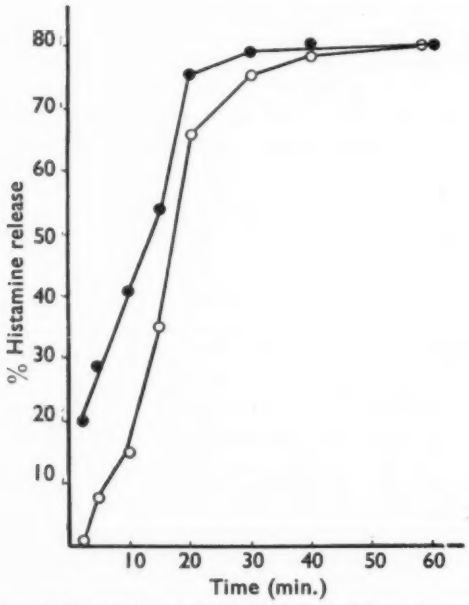

FIG. 2.—Effect of time of incubation on histamine release by antihistamines, diphenhydramine 0.001 M ●——● and mepyramine 0.001 M O——O, from guinea-pig lung. Ordinates: histamine release expressed as a % of the total tissue histamine. Abscissae: time in min. Each point is mean of 2 experiments.

from guinea-pig lung, diaphragm, mesentery, and skin, very little from the ileum and practically nothing from the stomach.

Effect of the Concentration of Antihistamines.—To study the strength of antihistamines as histamine releasers lung tissue was incubated with different concentrations of bromodiphenhydramine, diphenhydramine, and mepyramine and the amount of histamine released by each concentration determined. The results, summarized in Fig. 1, show that histamine release is related to the concentration of the antihistamine.

Effect of Time of Incubation with Antihistamines on Histamine Release.—Pieces of lung tissue were incubated with antihistamines for periods of 2 min. up to 60 min. The results are shown in Fig. 2. A significant release of histamine occurred as early as 2 min. after incubation with diphenhydramine and 5 min. with mepyramine, a maximal release being attained after 40 min. It was not possible to release more than 80% of the total histamine even when the time of incubation was increased to 2 hr.

Effect of p*H on Histamine Release by Antihistamines.*—To study the effect of *p*H on histamine release by antihistamines, samples of guinea-pig lung were incubated with diphenhydramine and mepyramine in Tyrode solution containing phosphate buffer to give *p*H values ranging from 6.5 to 8.5. Results are shown in Fig. 3. It was observed that the release of histamine by antihistamines was dependent upon *p*H, being greater at *p*H 8.5 and negligible at *p*H 6.5.

Effect of Temperature.—The effect of temperature was observed by incubating lung tissue with antihistamines for 15 min. at different temperatures

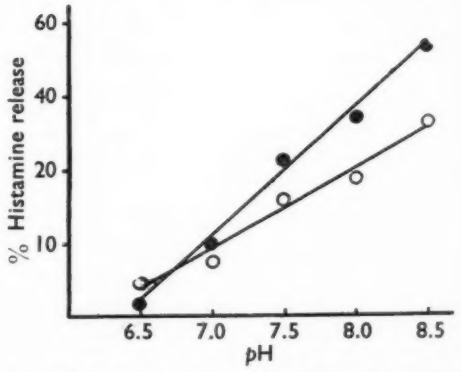

FIG. 3.—Effect of *p*H on histamine release by antihistamines, diphenhydramine 0.001 M ●——● and mepyramine 0.001 M O——O, from guinea-pig lung. Ordinates: histamine release is expressed as a % of the total tissue histamine. Abscissae: *p*H. Each point is mean of 2 experiments.

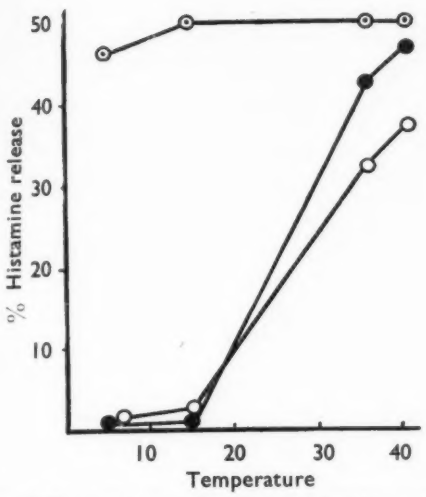

FIG. 4.—Effect of temperature on histamine release by antihistamines, bromodiphenhydramine 0.001 M ⊙——⊙, diphenhydramine 0.001 M ●——●, and mepyramine 0·001 M O——O, from guinea-pig lung. Ordinates: histamine release expressed as a % of the total tissue histamine. Abscissae: temperature. Each point is mean of 2 experiments.

ranging from 5° to 42°. Diphenhydramine and mepyramine were inactive at low temperatures, but bromodiphenhydramine was as active at 5° as at 42° (Fig. 4).

Effect of Metabolic Inhibitors, Calcium Lack and High Temperature on Histamine Release by Antihistamines.—It is known that various metabolic inhibitors, calcium lack or previous heating of the tissue at 45° inhibit histamine release in anaphylaxis, but potentiate histamine release by chemical histamine-liberators in the guinea-pig (Mongar and Schild, 1957a, 1957b, 1958). It was therefore of interest to study the effect of such treatments on histamine release by antihistamines. Lung tissue was incubated for 15 min. at 37° with either 0.001 M sodium iodoacetate, 0.001 M *N*-ethylmaleimide or calcium-free Tyrode solution containing 0.01% sodium edetate. Some samples were previously heated at 45° for 15 min. and brought back to 37°. After these treatments bromodiphenhydramine 50 μg./ml., diphenhydramine 100 μg./ml., or mepyramine 500 μg./ml. was added and the tissue was incubated for a further period of 15 min. The histamine release was then determined. Iodoacetate and *N*-ethylmaleimide potentiated histamine release by antihistamines (an average of 150% increase in 2 experiments), while calcium lack and previous heating at 45° were either without effect or only slightly potentiated histamine release by antihistamines.

Mast Cell Damage.—When pieces of guinea-pig mesentery were incubated with antihistamines in Tyrode solution at 37° damage to the mast cells occurred. Cytologically the mast cell damage induced by antihistamines was very similar to that induced by octylamine (Mota, 1959); characterized by diffusion of the metachromatic material and later dissolution of the cell. All the antihistamines tested induced the same kind of mast cell damage and caused a decrease in the number of these cells (Table II). Paralleling the histamine release this damage increased with time of incubation and concentration of the drug, and was *p*H and temperature dependent. Furthermore, iodoacetate, *N*-ethylmaleimide, calcium lack or previous heating at 45° potentiated the action of the antihistamines considerably. A concentration of antihistamine

TABLE II

MAST CELL CONTENT OF GUINEA-PIG MESENTERY INCUBATED AT 37° FOR 15 MIN. IN TYRODE SOLUTION WITH AND WITHOUT ADDED ANTIHISTAMINE

Each figure is the mean count of 30 microscopical fields (magnification 130) in each of 2 experiments.

Antihistamine	Concentration μg./ml.	Number of Mast Cells in		% Reduction
		Tyrode Alone	Tyrode with Antihistamine	
Chlorpheniramine maleate	50	12	13	0
	100	12	2	83
Bromodiphenhydramine hydrochloride	12·5	15	14	0
	25	15	3	78
Isothipendyl hydrochloride	25	19	20	0
	50	19	1	94
Antazoline hydrochloride	25	21	21	0
	50	21	4	81
Diphenhydramine hydrochloride	50	14	14	0
	100	14	1	93
Methapyrilene hydrochloride	100	17	18	0
	200	17	2	88
Mepyramine hydrogen maleate	200	24	25	0
	500	24	6	75
Promethazine hydrochloride	6·25	11	11	0
	12·50	11	3	73
Chlorpheniramine hydrochloride	100	31	31	0
	200	31	2	94
Tripelennamine hydrochloride	100	13	13	0
	200	13	3	77
Phenindamine hydrogen tartrate	25	27	29	0
	50	27	10	63

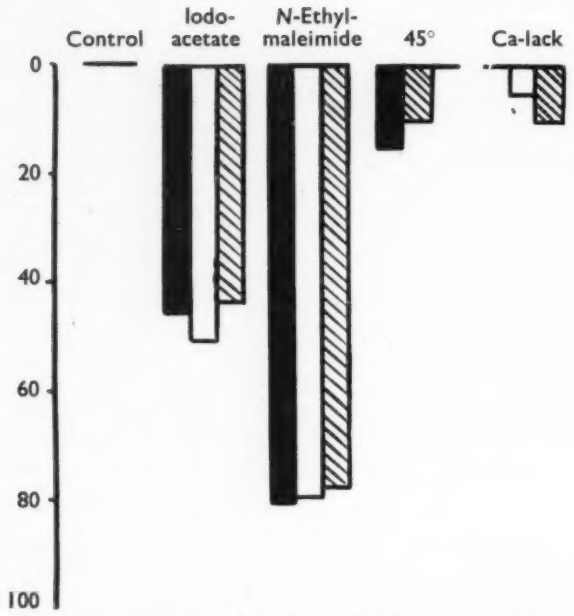

FIG. 5.—Effect of iodoacetate (0.001 M), *N*-ethyl-maleimide (0.001 M), calcium lack and previous heating at 45° on guinea-pig mast cell damage induced by bromodiphenhydramine (6.5 μg./ml.) ■, diphenhydramine (50 μg./ml.) ▩ and mepyramine (250 μg./ml.) □. Ordinates: % diminution in mast cell count. Control: the effect induced by the antihistamine alone.

incapable by itself of causing damage to the mast cells completely destroyed these cells when the tissue was previously submitted to any of these treatments (Fig. 5).

Inhibition of Histamine Release by Antigen.—When pieces of sensitized guinea-pig lung were previously incubated with antihistamines, the histamine release induced by later contact with antigen was reduced (Table III). The concentration of antihistamine necessary to produce a total inhibition of histamine release by antigen was very near to the concentration which itself released histamine. Concentrations of diphenhydramine and mepyramine able to release histamine by themselves always produced a total inhibition of histamine release by antigen. However, it was never possible to obtain a complete inhibition of histamine release in anaphylaxis with bromodiphenhydramine.

Inhibition of Mast Cell Damage by Antigen.—The minimal concentrations of antihistamines necessary to produce a total inhibition of mast cell reduction by antigen are shown in Table IV. When sensitized guinea-pig mesentery previously incubated with antihistamine was incubated with antigen, the usual mast cell diminution induced by antigen-antibody reaction did not occur.

TABLE III

EFFECT OF ANTIHISTAMINES ON HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80

Sensitized lung tissue was previously incubated with antihistamine, washed in Tyrode solution and transferred to antigen (1 mg./ml.) or to compound 48/80 (1 mg./ml.). Each figure is the mean of 2 experiments.

Antihistamine	Concentration μg./ml.	% Histamine Release by		% Inhibition
		Antihistamine	Antigen	
Bromodiphenhydramine hydrochloride	0	—	36	—
	5	0	34	0
	10	0	37	0
	15	0	31	14
	25	11	25	30
	30	21	23	36
Diphenhydramine hydrochloride	0	—	28	—
	25	0	30	0
	50	0	25	10
	100	11	6	78
	150	39	0	100
	200	49	0	100
Mepyramine hydrogen maleate	0	—	32	—
	25	0	31	0
	50	0	33	0
	100	0	20	38
	200	0	9·5	66
	250	0	0	100
	500	25	0	100
	800	55	0	100
			48/80	% Potentiation
Bromodiphenhydramine hydrochloride	0	—	14	—
	15	9	19	35
	30	26	25	78
Diphenhydramine hydrochloride	0	—	16	—
	100	9	20	25
	150	20	40	150
Mepyramine hydrogen maleate	0	—	16	—
	250	0	25	56
	500	20	28	75

Potentiation of Histamine Release and Mast Cell Damage by Compound 48/80.—It is known that in the guinea-pig several agents that inhibit histamine

TABLE IV

EFFECT OF ANTIHISTAMINES ON MAST CELL DAMAGE BY ANTIGEN OR COMPOUND 48/80

Sensitized guinea-pig mesentery was previously incubated with antihistamine, washed in Tyrode solution and transferred to antigen or to compound 48/80.

Antihistamine	Concentration μg./ml.	% Mast Cell Diminution by Antigen	% Inhibition
Chlorpheniramine maleate	0	50*	—
	50	0	100
Bromodiphenhydramine hydrochloride	0	37	—
	12·5	0	100
Isothiopendyl hydrochloride	0	60	—
	25	0	100
Antazoline hydrochloride	0	50	—
	25	0	100
Diphenhydramine hydrochloride	0	37	—
	50	0	100
Methapyrilene hydrochloride	0	68	—
	100	0	100
Mepyramine hydrogen maleate	0	37	—
	200	0	100
Promethazine hydrochloride	0	68	—
	6·25	0	100
Tripelennamine hydrochloride	0	50	—
	100	0	100
Phenindamine hydrogen tartrate	0	68	—
		0	100

Antihistamine	Concentration μg./ml.	% Mast Cell Diminution by 48/80	% Potentiation
Bromodiphenhydramine hydrochloride	0	28	—
	12·5	64	127
Diphenhydramine hydrochloride	0	32	—
	50	76	137
Mepyramine hydrogen maleate	0	48	—
	200	92	91

* Mean of 2 experiments.

release and mast cell damage in anaphylaxis potentiate the effects of chemical histamine releasers. We decided to study the effect of antihistamines on the histamine release and mast cell damage by compound 48/80. Lung tissue or mesentery were first incubated for 15 min. at 37° with Tyrode solution or Tyrode solution containing antihistamine, washed for 5 min. with Tyrode solution and then incubated for a further period of 15 min. at 37° with Tyrode solution containing 1 mg./ml. of compound 48/80. The results in Tables III and IV show that previous incubation with antihistamines potentiated histamine release and mast cell damage by compound 48/80.

TABLE V

HISTAMINE RELEASE *IN VITRO* BY ANTIHISTAMINES FROM RAT TISSUES

Histamine release is expressed as a % of the total tissue histamine. Each figure represents a different experiment.

Tissue	% Histamine Release by		
	Bromodiphenhydramine Hydrochloride 0·001 M	Diphenhydramine Hydrochloride 0·003 M	Mepyramine Hydrogen Maleate 0·003 M
Skin ..	10 11 13 6	9 15 10	8 9 11 6
Diaphragm	60 25 69	65 60 34	38 49 30 53
Stomach ..	6 7 2	1 2 3	0 2 5 0
Ileum ..	18 13 11	13 11 6	10 14 15 12
Mesentery	28 23 6 0	30 28 21 6	20 25 20 3

Experiments with Rat Tissues

Histamine Release from Various Rat Tissues.—Incubation of rat tissue with antihistamines led to histamine liberation (Table V). The antihistamines were particularly active on the rat diaphragm but had practically no effect on the histamine content of the stomach. The concentrations of antihistamines required to release histamine from rat tissues were higher than those needed to release histamine from guinea-pig tissues.

TABLE VI

MAST CELL DAMAGE *IN VITRO* BY ANTIHISTAMINES

Rat mesentery was incubated at 37° for 15 min. with antihistamines and the % of mast cells presenting granule extrusion determined. Each column represents a different animal.

Antihistamine	Concentration μg./ml.	Mast Cell Disruption
Bromodiphenhydramine hydrochloride	1,000	100 56 0 25 100
	500	0 0 37 100
	250	0 0 0
Diphenhydramine hydrochloride	1,000	100 100 20 0 0
	500	0 100 11 0 20
	250	0 0 0 0 0
Mepyramine hydrogen maleate	1,000	0 100 25 0 0
	500	0 0 0 0 0

TABLE VII

INHIBITION BY ANTIHISTAMINES OF HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80

Sensitized rat tissue was previously incubated with antihistamines, washed in Tyrode solution and transferred to antigen (10% horse serum) or to compound 48/80 (0·1 mg./ml.). Each figure represents the mean of 2 experiments.

Antihistamine	Concentration μg./ml.	Tissue	% Histamine Release by Antihistamine	% Histamine Release by Antigen	% Inhibition
Bromodiphenhydramine hydrochloride	0	Skin	—	13	—
	500		10	7	46
	250		10	11	15
	100		0	14	0
Diphenhydramine hydrochloride	0	Skin	—	11	—
	1,000		0	5	55
	500		0	6	45
	250		0	8	27
	50		0	11	0
	0	Mesentery	—	40	—
	1,000		30	3	93
	500		15	4	90
	100		0	39	0
Mepyramine hydrogen maleate	0	Skin	—	13	—
	1,000		0	0	100
	500		0	4	69
	250		0	6	54
	100		0	14	0
	0	Diaphragm	—	29	—
	500		0	9	69
	0	Mesentery	—	28	—
	500		0	14	50
				% Histamine Release by 48/80	
Bromodiphenhydramine hydrochloride	0	Skin	—	16	—
	500		5	10	37
	250		0	12	25
	100		0	17	0
Diphenhydramine hydrochloride	0	Skin	—	16	—
	1,000		6	6	63
	500		0	9	44
	250		0	10	37
	100		0	16	0
Mepyramine hydrogen maleate	0	Skin	—	36	—
	1,000		0	0	100
	500		0	14	61
	250		0	23	36
	100		0	37	0

TABLE VIII

EFFECT OF ANTIHISTAMINES ON MAST CELL DISRUPTION BY ANTIGEN OR COMPOUND 48/80

Sensitized rat mesentery was first incubated with antihistamine, washed in Tyrode solution and then transferred to antigen (10% horse serum) or to compound 48/80 (0.1 mg./ml.).

Antihistamine	Concentration μg./ml.	% Mast Cell Disruption by Antigen	% Inhibition
Bromodiphenhydramine hydrochloride	0	26*	—
	500	13	50
	250	21	19
	50	28	0
Diphenhydramine hydrochloride	0	60	—
	1,000	0	100
	500	0	100
	250	35	42
	100	59	0
Mepyramine hydrogen maleate	0	49	—
	1,000	0	100
	500	0	100
	250	20	59
	100	53	0
		% Mast Cell Disruption by Compound 48/80	
Bromodiphenhydramine hydrochloride	0	62	—
	500	20	68
	250	60	0
	100	63	0
Diphenhydramine hydrochloride	0	76	—
	1,000	0	100
	500	0	100
	250	75	0
	100	79	0
Mepyramine hydrogen maleate	0	80	—
	1,000	0	100
	500	0	100
	250	70	13
	100	81	0

* Mean of 2 experiments.

Rat Mast Cell Damage.—All the antihistamines tested caused mast cell disruption similar to that described with compound 48/80 (Mota, Beraldo, and Junqueira, 1953). However, concentrations as high as 1 mg./ml. were frequently necessary to produce 100% mast cell disruption. Even so, this disruption was less conspicuous than that induced by 0.1 mg./ml. compound 48/80. The results with mepyramine, bromodiphenhydramine, and diphenhydramine are shown in Table VI. There was a large individual variation in the response of the mast cells to the action of the antihistamines. For example, mepyramine 1 mg./ml. in some animals produced 100% mast cell disruption and in others no disruption at all.

Using phase contrast microscopy it was observed that isolated mast cells responded to antihistamines by the sudden appearance of intracellular vacuoles and transient swelling of the cell. Simultaneously extrusion of granules was frequently seen.

Inhibition of Histamine Release by Antigen or Compound 48/80.—As with sensitized guinea-pig tissue, previous incubation of rat tissue with antihistamines caused a total or partial inhibition of histamine release in anaphylaxis (Table VII). However, higher concentrations of antihistamine were necessary to inhibit histamine release in rat anaphylaxis than in guinea-pig anaphylaxis. Antihistamines also inhibited the histamine release induced by compound 48/80 in the rat.

Inhibition of Mast Cell Damage by Antigen or Compound 48/80.—When rat mesentery was first incubated with antihistamines and then transferred to antigen (10% horse serum) or to compound 48/80 (0.1 mg./ml.) mast cell disruption was prevented (Table VIII). Concentrations of antihistamines that disrupted mast cells by themselves were avoided in these experiments since it was found difficult to distinguish the disruption induced by antihistamines from that induced by compound 48/80 when both were simultaneously present.

Discussion

The release of histamine from tissues by antihistamines was observed by Arunlakshana (1953a) and more recently by Tasaka (1957). This may explain the reported rise in human blood histamine after treatment with antihistamines (Pellerat and Murat, 1946). It has also been observed that mesenteric mast cells are destroyed after repeated intraperitoneal injections of promethazine (Smith, 1958a).

It is conceivable that antihistamines compete with histamine not only at its site of action, the histamine receptor, but also at its site of storage in the cell. If this is so it might be expected that the histamine-releasing activity and antihistamine activity of different compounds would be correlated.

In the present investigation the antihistamines behaved as typical histamine liberators; their effects increased with concentration, *p*H and temperature. Their effects were potentiated by pre-treatment with metabolic inhibitors like those of octylamine and compound 48/80 (Mongar and Schild, 1957a, 1957b; Mota, 1959). Different antihistamines differed markedly in histamine-releasing and mast cell destroying activity. We found that bromodiphenhydramine and promethazine were the most potent histamine releasers. There was also a considerable difference between different tissues. The tissues of the digestive tract released only small amounts of histamine. There was an interesting quantitative difference between the effects of the antihistamines and of the compound 48/80 in the rat and guinea-pig. In the guinea-pig the antihistamines were more active as histamine releasers than compound 48/80, and even more active than the relatively potent octylamine, judging from the data of Mongar and Schild (1953). In the rat, however, the antihistamines are less active than compound 48/80.

The observation that antihistamines act on isolated mast cells in the absence of plasma or tissue fluid suggests that they act directly on the cell. The response of the rat mast cell to antihistamines is morphologically similar to that produced by a specific antigen (Mota, Dias da Silva, and Ferreira Fernandes, 1960a).

The mechanism of the inhibitory effect of antihistamines on histamine release in anaphylaxis is not clear. Arunlakshana (1953b), who was the first to observe an inhibition of histamine release in anaphylaxis by antazoline, considered that it was due to the release, by the antihistamine, of a large proportion of the available tissue histamine, and therefore the amount releasable by antigen was diminished. Our findings suggest that this cannot be the whole explanation. With several of the antihistamines the dose which produced inhibition of histamine release in anaphylaxis was definitely less than that which produced histamine release on its own. Moreover, the histological evidence showing that mast cells fail to be disrupted by specific antigen after pre-treatment with an antihistamine demonstrates that a true protective action is being exerted. It is possible that antihistamine molecules in a critical concentration near to that required for histamine release become attached to the mast cell membrane and interfere with the antigen antibody reaction. Alternatively

they may inhibit the enzymatic system (Mongar and Schild, 1957a) required in the anaphylactic reaction.

There is no evidence that the histamine-releasing property of antihistamines and their property of preventing histamine release in anaphylaxis are correlated. For example, bromodiphenhydramine in a concentration that released histamine by itself did not completely inhibit histamine release in anaphylaxis, whereas mepyramine completely blocked an anaphylactic histamine release in concentrations which did not themselves release histamine. Furthermore, compound 48/80 in a concentration which releases a small amount of histamine does not inhibit but, on the contrary, potentiates anaphylactic histamine release (Mongar and Schild, 1952).

The concentration of antihistamines necessary to prevent a diminution in mast cell count by the specific antigen completely was frequently smaller than the concentration for complete inhibition of histamine release. For example, 100 μg./ml. mepyramine completely prevented a mast cell decrease by antigen but produced only a partial reduction of histamine release. This discrepancy may be due to qualitative alterations of mast cells which were not considered in our measurements, and also to the possible release of histamine from mast cells before any visible morphological change occurs (Smith, 1958b). The observation that antihistamines inhibited histamine release and mast cell damage by compound 48/80 in rat tissues agrees with previous results showing that the various agents that potentiate histamine release and mast cell damage by compound 48/80 in guinea-pig tissues inhibit these same phenomena when acting on rat tissues (Mota and Ishii, 1960).

The inhibition of histamine release in anaphylaxis by antihistamines may help to explain their protective action in anaphylaxis. In some cases at least the protection may be due more to inhibition of mast cell disruption than to competition at the receptor site. This, however, probably does not apply to the protective effect exerted by antihistamines in the anaphylactic shock of the intact guinea-pig, as concentrations of antihistamines necessary to inhibit histamine release *in vitro* are much higher than those likely to be produced by protective doses of antihistamines *in vivo*. On the other hand it is possible that the reduction in passive cutaneous anaphylaxis by high doses of antihistamines in rats (Brocklehurst, Humphrey, and Perry, 1955) may be due in part at least to the anti-anaphylactic effect of such doses of antihistamines. This inhibition of histamine release may possibly be attended by simultaneous inhibition of the release of other pharmacologically active substances, since Brocklehurst (1958) has shown that inhibition of histamine release in anaphylaxis is usually accompanied by inhibition of SRS-A release. The so-called unspecific effects of antihistamines in isolated tissues (Hawkins and Rosa, 1956) may also be explainable at least partially by their anti-anaphylactic actions.

As a protective agent in anaphylaxis mepyramine appears to be safer than bromodiphenhydramine or diphenhydramine as the ratio between its protective concentration and the concentration that releases histamine is greater. Mepyramine may owe its strong anti-anaphylactic action to the pyridine nucleus in its molecule, since we have observed (Mota, Dias da Silva, and Ferreira Fernandes, 1960b) that pyridine and some of its derivatives are potent inhibitors of histamine release in anaphylaxis.

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THE INHIBITION OF MAST CELL DAMAGE AND HISTAMINE RELEASE IN ANAPHYLAXIS BY PYRIDINE AND DIPHOSPHOPYRIDINE NUCLEOTIDASE INHIBITORS. COMPARISON WITH COMPOUND 48/80

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In rats and guinea-pigs both mast cell damage and histamine release in anaphylaxis were inhibited by pyridine, nicotinamide, diethylnicotinamide, nicotinic acid, isonicotinic acid and isonicotinic acid hydrazide, which are known to be inhibitors of diphosphopyridine nucleotidases. On the other hand these compounds potentiated histamine release and mast cell damage by compound 48/80 in guinea-pigs although inhibiting the same phenomena in rats. The relationship of these findings with the mechanism of histamine liberation in anaphylaxis is discussed.

The isolation and synthesis of a dinucleotide of histamine was recently reported by Alivisatos (1958a). The structure of this compound is that of diphosphopyridine nucleotide (DPN) in which histamine replaced the nicotinamide moiety. Its biosynthesis involves a simple exchange reaction between free histamine and the nicotinamide of DPN. Alivisatos suggested that the exchange reaction of DPN with histamine could be involved in the mechanism of anaphylaxis. As this biosynthesis takes place in the presence of diphosphopyridine nucleotidase (DPN-ase), it was of interest to investigate the effect of compounds known to inhibit the activity of this enzyme on the mast cell damage and histamine release which occur in anaphylaxis. In addition, the effects of these compounds on the mast cell damage and histamine release by compound 48/80 were also investigated.

METHODS

Wistar rats and guinea-pigs of either sex and body weight 200 to 400 g. were used in all experiments. Guinea-pigs were sensitized with a 2% solution of egg albumen in 0.5% aqueous phenol, of which 1 ml. was injected subcutaneously and 1 ml. intraperitoneally. The animals were used from 3 weeks after sensitization. Rats were sensitized with 1 ml. of horse serum plus 1 ml. of *Haemophilus pertussis* vaccine, containing 20,000 million organisms, administered intraperitoneally. Evidence of sensitization in both species was provided by testing whether mast cells presented damage in the presence of the specific antigen (Mota, 1959 ; Humphrey and Mota, 1959; Mota and Ishii, 1960). Pyridine and the following DPN-ase inhibitors were used: nicotinamide, nicotinic acid, isonicotinic acid, isonicotinic acid hydrazide and diethylnicotinamide. These substances were dissolved in Tyrode solution and when necessary the *p*H was adjusted to 7.8 with sodium hydroxide or hydrochloric acid. Histamine release was measured in rat skin or guinea-pig lung and mast cells were observed in the mesentery. The animals were killed with a blow on the head and bled from the jugular veins. Rat skin was shaved with an electric clipper, cut in pieces of about 1 sq. cm., carefully removed from the abdominal wall and kept in cold Tyrode solution. One skin piece was used as a sample. Guinea-pig lung was cut in slices 0.8 mm. thick with a McIlwain tissue chopper. The slices were washed in cold Tyrode solution for about 1 hr. and 3 or 4 of them were used as a sample. Rat or guinea-pig mesentery was dissected out from the small intestine and cut into several small pieces, which were dipped into cold Tyrode solution. Three or four of these pieces were used as a sample. To study the effect of pyridine and DPN-ase inhibitors on mast cell damage and histamine release by antigen or compound 48/80, rat or guinea-pig tissue samples were shaken gently in 50 ml. beakers closed with parafilm caps for a standard time of 15 min. at 37° in 4 ml. of Tyrode solution containing different concentrations of the substances to be tested. After this treatment either antigen or compound 48/80 was added and the tissues incubated for a further period of 15 min. after which the mesentery was transferred to fixative and the skin or lung samples were transferred to N-hydrochloric acid, cut in very small pieces and boiled for 2 or 3 min. for histamine extraction.

The incubation fluid was acidified with one drop of concentrated hydrochloric acid, boiled and kept at 4° until assayed. As a control to each experiment a tissue

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TABLE I

EFFECT OF PYRIDINE AND DPN-ASES INHIBITORS ON GUINEA-PIG MAST CELL DIMINUTION INDUCED BY ANTIGEN OR COMPOUND 48/80

Histamine-releasing Agent	Molar Concentration of Chemicals	% Diminution by: Releasing Agent Alone	% Diminution by: Releasing Agent Plus Chemical	% Inhibition (−) or Potentiation (+)
Antigen	Diethylnicotinamide 0·040	53	0	−100
	0·040	61	0	−100
	0·020	58	0	−100
	0·010	41	20	− 51
	0·001	37	32	− 13
Antigen	Nicotinamide 0·040	80	0	−100
		59	0	−100
	0·020	59	25	− 57
	0·010	59	40	− 32
	0·005	59	40	− 32
	0·001	27	28	0
48/80	0·040	55	100	+ 81
		40	40	0
		25	60	+140
		40	56	+ 40
		70	95	+ 36
Antigen	Nicotinic acid 0·040	81	41	− 49
		88	42	− 52
		94	56	− 40
		51	31	− 39
		63	30	− 52
48/80	0·040	40	56	+ 40
		31	90	+190
		41	52	+ 26
		36	36	0
Antigen	Isonicotinic acid 0·040	28	28	0
		55	42	− 24
		76	0	−100
		55	38	− 31
48/80	0·040	41	65	+ 59
		60	100	+ 67
Antigen	Pyridine 0·040	37	26	− 30
		48	20	− 58
48/80	0·040	33	64	+ 94
		66	66	0
		54	78	+ 44
		61	62	0
Antigen	Isonicotinic acid hydrazide 0·040	75	32	− 57
		81	55	− 32
		45	45	0
48/80		55	100	+ 81
		21	25	+ 19
		38	38	0

TABLE II

EFFECT OF PYRIDINE AND DPN-ASES INHIBITORS ON HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80 FROM GUINEA-PIG LUNG

Histamine release is expressed as % of the total tissue histamine.

Releasing Agent	Molar Concentration of Chemicals	% Histamine Release by: Releasing Agent Alone	% Histamine Release by: Releasing Agent Plus Chemical	% Inhibition (−) or Potentiation (+)
Antigen	Diethylnicotinamide 0·040	19	0	−100
		15	0	−100
		44	0	−100
		31	1	− 97
		31	3	− 90
48/80		28	28	0
		28	32	+ 14
		21	21	0
Antigen	Nicotinamide 0·040	22	0	−100
		27	0	−100
		28	8	− 71
		39	9	− 77
48/80	0·040	10	16	+ 60
		21	32	+ 52
		26	26	0
Antigen	Nicotinic acid 0·040	33	11	− 67
		15	4	− 73
		19	12	− 36
48/80		21	33	+ 57
		23	23	0
		45	45	0
Antigen	Isonicotinic acid 0·040	34	24	− 29
		31	12	− 61
		25	8	− 68
48/80		28	37	+ 32
		16	24	+ 50
		17	17	0
Antigen	Pyridine 0·040	34	23	− 32
		30	18	− 40
		31	20	− 35
48/80		21	27	+ 28
		17	17	0
Antigen	Isonicotinic acid hydrazide 0·040	34	30	− 12
		44	32	− 27
		28	20	− 28
48/80		21	29	+ 38
		27	27	0

sample of similar size and thickness was incubated in Tyrode solution alone. At the end of the experiments the tested compound was added to the incubation fluids so that all of them contained the same concentration of the compound. Thus, any possible difference in the histamine level due to interference of the tested compound with the response of the ileum to histamine during the assay was eliminated. To assay histamine the extracts and incubation fluids were neutralized with NaOH and when necessary diluted with Tyrode solution to the required volume. Histamine was assayed on the atropinized guinea-pig ileum. In order to detect any non-histamine component the extracts and incubation fluids were reassayed after inhibition of histamine by mepyramine 10^{-8}. Histamine loss in controls was subtracted from that released by antigen or compound 48/80. Histamine release is expressed as a percentage of the total tissue histamine. Rat mesentery was fixed and stained in a 10% formaldehyde solution containing 1% toluidine blue and 0.1% acetic acid; guinea-pig mesentery was fixed in a 4% solution of lead sub-acetate in 50% ethanol, plus 1% of acetic acid (Mota and Vugman, 1956). The mesentery was observed as a whole-mount preparation. Rat mast cell damage was assessed by counting the percentage of cells presenting granules extrusion, 500 cells being counted; guinea-pig mast cell damage was assessed by counting the total number of metachromatic granules containing cells in 30 microscopical fields using a magnification of 120.

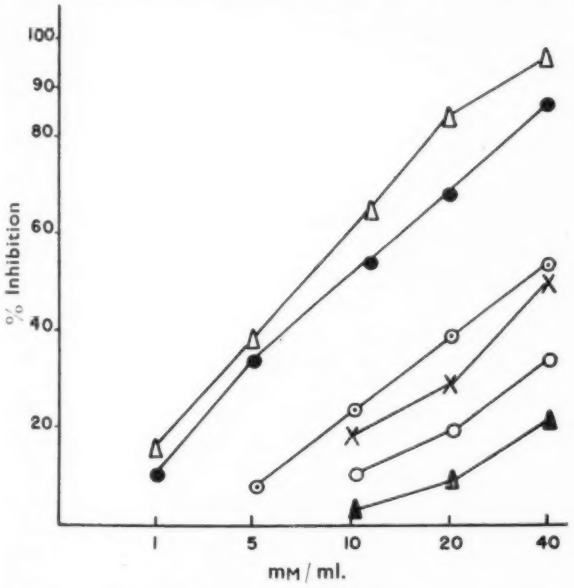

FIG. 1.—Effect of different concentrations of pyridine (O——O) and DPN-ase inhibitors (nicotinamide ●——●, nicotinic acid X——X, diethylnicotinamide Δ——Δ, isonicotinic acid hydrazide ▲—▲, isonicotinic acid ⊙——⊙) on histamine release by antigen from guinea pig lung. Ordinates: %. Inhibition was calculated from the difference between the amount of histamine released by antigen plus inhibitor and that released by antigen alone. Abscissae: concentration of inhibitor. Each point is mean of 2 experiments.

RESULTS

Effect of Pyridine and Inhibitors of Diphosphopyridine Nucleotidase on Mast Cell Damage and Histamine Release by Antigen from Sensitized Guinea-pig Tissues.—When sensitized guinea-pig lung or mesentery was previously incubated with 0.04 M pyridine, diethylnicotinamide, nicotinamide, nicotinic acid, isonicotinic acid and isonicotinic acid hydrazide, the subsequent mast cell damage and histamine release by antigen was greatly reduced as shown in Tables I and II. Diethylnicotinamide was the most effective inhibitor of mast cell damage and histamine release, followed closely by nicotinamide. Isonicotinic acid hydrazide was the weakest inhibitor of all.

It was also observed that this inhibition was directly proportional to the concentration of these compounds present (Fig. 1). When nicotinamide was added simultaneously with antigen to the sensitized tissue, the subsequent histamine release was markedly reduced, indicating that it acts very quickly in inhibiting histamine release (Fig. 2). A maximal inhibitory effect was seen after 15 min.

Effect of Pyridine and DPN-ase Inhibitors on Mast Cell Damage and Histamine Release by Compound

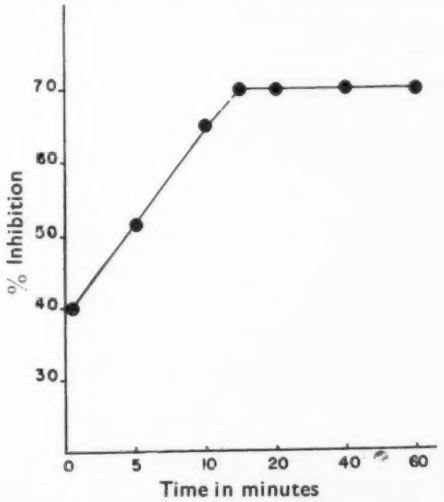

FIG. 2.—Effect of time of pre-incubation with nicotinamide (0.020 M) on the subsequent inhibition of histamine release by antigen. At 0 min. the antigen was added simultaneously with nicotinamide to the tissue. Ordinates: %. Inhibition calculated from the difference between the amount of histamine released by antigen plus inhibitor and that released by antigen alone. Abscissae: time in min. Each point is mean of 2 experiments.

TABLE III

INHIBITION BY PYRIDINE AND DPN-ASES INHIBITORS OF RAT MAST CELL DISRUPTION INDUCED BY ANTIGEN OR COMPOUND 48/80

Releasing Agent	Molar Concentration of Chemicals	% Disruption by Releasing Agent Alone	% Disruption by Releasing Agent Plus Chemical	% Inhibition
Antigen	Diethylnicotinamide 0·040	67	0	100
		31	0	100
		56	0	100
		48	15	69
48/80	0·040	36	0	100
		77	0	100
		73	60	18
		29	21	28
Antigen	Nicotinamide 0·040	36	0	100
		45	20	56
		64	0	100
		13	0	100
48/80	0·040	31	10	68
		89	0	100
		44	21	52
		35	30	14
Antigen	Isonicotinic acid 0·040	26	0	100
		33	0	100
		64	10	84
		28	0	100
48/80	0·040	68	34	50
		71	35	50
		60	0	100
Antigen	Nicotinic acid 0·040	56	0	100
		58	34	41
		20	0	100
		29	20	31
48/80	0·040	90	20	78
		87	40	54
		76	76	0
Antigen	Isonicotinic acid hydrazide 0·040	38	20	47
		49	0	100
		34	34	0
		16	10	38
48/80	0·040	81	81	0
		73	73	0
		98	98	0

TABLE IV

INHIBITION BY PYRIDINE AND DPN-ASES INHIBITORS OF HISTAMINE RELEASE BY ANTIGEN OR COMPOUND 48/80 FROM SENSITIZED RAT SKIN

Histamine release is expressed as % of the total tissue histamine.

Releasing Agent	Molar Concentration of Chemicals	% Histamine Release By Releasing Agent Alone	% Histamine Release By Releasing Agent Plus Chemical	% Inhibition
Antigen	Diethylnicotinamide 0·040	12·8	2·7	78
	0·040	13	0	100
	0·040	7·8	0	100
	0·020	7·8	2·4	69
	0·010	7·8	3	61
	0·001	7·8	7·9	0
48/80	0·040	8	8	0
		14	14	0
		31	19	39
		18	9	50
Antigen	Nicotinamide 0·040	12·8	0	100
	0·040	16	0	100
	0·040	7·8	0	100
	0·025	7·8	2	75
	0·020	7·8	0	100
	0·010	7·8	3·6	54
	0·001	7·8	7·8	0
48/80	0·040	14	14	0
		20	9	55
		31	26	16
		18	10	44
		11	11	0
Antigen	Isonicotinic acid 0·040	12·8	3·8	70
		16	0	100
	0·020	16	3	81
	0·010	16	12	25
	0·005	16	14	12
	0·001	16	16	0
48/80	0·040	18	7	61
		31	15	52
		8	5	38
		14	9	36
		20	14	30
Antigen	Isonicotinic acid hydrazide 0·040	12·8	5·8	55
		16	14	12
	0·020	16	12	25
48/80	0·040	14	14	0
		20	20	0
		31	31	0
		16	10	27

48 80.—It is known that in the guinea-pig all the substances which inhibit histamine release and mast cell damage by antigen potentiate the effects of chemical histamine releasers. It was therefore of interest to investigate the effect of pyridine and DPN-ase inhibitors on the mast cell damage and histamine release by compound 48/80. The results of these experiments are shown in Tables I and II. The mast cell damage and histamine release by compound 48/80 were potentiated by all the compounds studied.

Effect of Pyridine and DPN-ase Inhibitors on Mast Cell Damage and Histamine Release by Antigen or Compound 48/80 *from Sensitized Rat Tissues.*—The results of these experiments are summarized in Tables III and IV. When rat skin or mesentery was left for 15 min. in contact with pyridine or any of the DPN-ase inhibitors studied, the subsequent mast cell damage and histamine release by antigen or compound 48/80 was reduced. Both nicotinamide and diethylnicotinamide were very effective inhibitors of mast cell damage and histamine release in anaphylaxis but less effective inhibitors of mast cell damage and histamine release by compound 48/80. Isonicotinic acid was the most effective compound in preventing histamine release by compound 48/80.

Discussion

Inhibition of mast cell damage and histamine release in anaphylaxis has been obtained with a large number of unrelated substances (Mongar and Schild, 1957; Mota, 1959; Mota and Ishii, 1960), most of which are toxic enough to prevent their use *in vivo*. These new inhibitors of anaphylaxis are outstanding for their low toxicity. In this respect nicotinamide is the most interesting compound since it has a very high therapeutic index and so can be safely used in patients. However, it will be necessary to test nicotinamide *in vivo* under the more complex conditions of the anaphylactic reaction in the intact animal. It will also be necessary to test whether pyridine and the other compounds here studied inhibit cellular metabolism in general, since so far the chemical inhibitors of anaphylaxis have been found to depress other cellular functions, too (Mongar and Schild, 1957).

It is interesting that nicotinamide is an essential constituent of diphosphopyridine nucleotide and triphosphopyridine nucleotide and that the other compounds studied are analogues of the vitamin which might interfere with the synthesis of breakdown of the coenzymes containing nicotinamide. The ability of diphosphopyridine nucleotidase to catalyse an exchange reaction between the nicotinamide moiety of diphosphopyridine nucleotide and histamine was recently reported by Alivisatos (1958a, 1958b). This author suggested that histamine released in anaphylaxis would combine with DPN, in presence of DPN-ase, displacing nicotinamide. This exchange reaction would be inhibited by nicotinamide and similar compounds due to the well-known inhibitory effect of nicotinamide on animal DPN-ases (Zatman, Kaplan and Colowick, 1953). However, if following its release in anaphylaxis, histamine becomes bound to DPN, in presence of nicotinamide, which inhibits this exchange reaction, more free histamine should be found in the incubation fluid. This does not agree with our present findings that nicotinamide and other compounds known to inhibit DPN-ases inhibited histamine release by antigen. Nevertheless, DPN-ases may be implicated in the mechanism of histamine release in anaphylaxis. Thus, histamine may be bound to the tissues in a way that requires the activity of DPN-ases for its release. In this case, nicotinamide or any other inhibitor of DPN-ases would inhibit histamine release by antigen.

The observation that mast cell damage and histamine release by compound 48/80 in the guinea-pig are potentiated by DPN-ase inhibitors suggests that the mechanism through which histamine is released by compound 48/80 is different from that acting in anaphylaxis, as already concluded by Mongar and Schild (1957). However, it seems that if the same compound which inhibits histamine release in anaphylaxis potentiates histamine release by compound 48/80, the mechanism of histamine release in the two cases, although certainly different, must be in some way related. It seems interesting to point out here that in rats histamine release by either antigen or compound 48/80 is inhibited. This has already been observed in connexion with other inhibitors of anaphylaxis in the rat (Mota and Ishii, 1960) and suggests that in this species the mechanism of histamine release by compound 48/80 is closer to that of antigen that in the guinea-pig.

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THE ISOLATED CHICK BIVENTER CERVICIS NERVE-MUSCLE PREPARATION

BY

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The isolated biventer cervicis nerve-muscle preparation can be used to distinguish between neuromuscular blocking agents which cause depolarization and those which do not. Both reduce the contractions caused by nerve stimulation, but depolarizing drugs also cause a contracture of the muscle.

Child and Zaimis (1954), Child (1955) and Tyler (1960) have described the use of certain muscles of the chick, the semispinalis cervicis and the biventer cervicis, as isolated preparations. Like the rectus abdominis of the frog, these respond to the application of depolarizing substances by giving a contracture.

We have found it possible to obtain a nerve-muscle preparation by using the innervated lower belly of the biventer cervicis whose nerve supply is enclosed by the tendon between the two bellies. A stimulus applied via electrodes in contact with this tendon results in a contraction of the muscle. The preparation may therefore be used to test simultaneously both for neuromuscular blocking activity, as indicated by a reduction in the contraction produced by nerve stimulation, and for depolarizing activity, as indicated by contracture. This preparation was demonstrated to the Physiological Society in July, 1959.

METHODS

Chickens (50 to 250 g.) were anaesthetized with sodium phenobarbitone (9% solution in water; 0.2 ml./100 g.) injected into a wing vein. The dissection was similar to that described by Child (1955) (compare Tyler, 1960). The back of the neck was plucked and the skin incised along the midline from the skull to below the base of the neck, exposing the two biventer cervicis muscles on either side of the midline and immediately below the skin. A thread was tied round the upper belly of one muscle which was then cut free from its attachment to the skull. When the thread was gently pulled the tendon joining the two bellies of the muscle and the lower (caudal) belly of the muscle could be identified and separated from the underlying semispinalis cervicis

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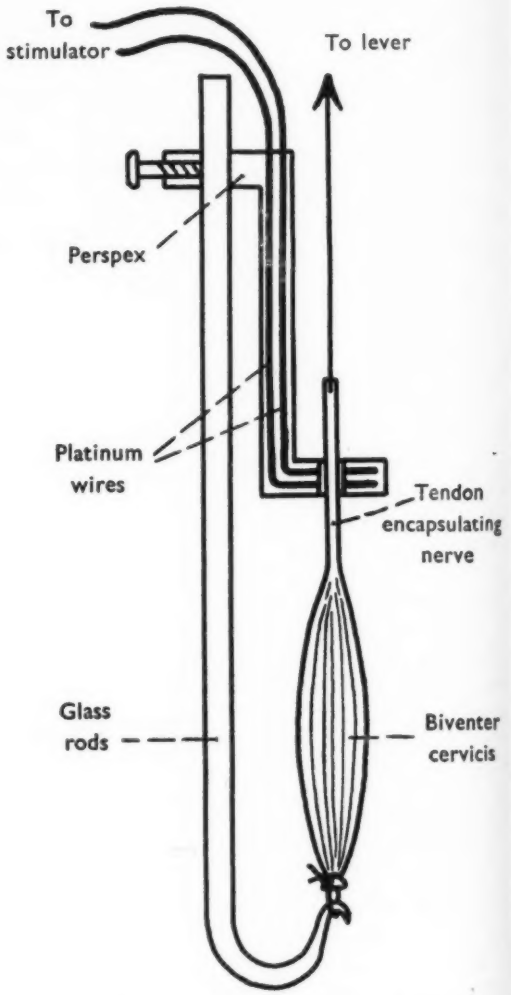

FIG. 1.—Electrode assembly.

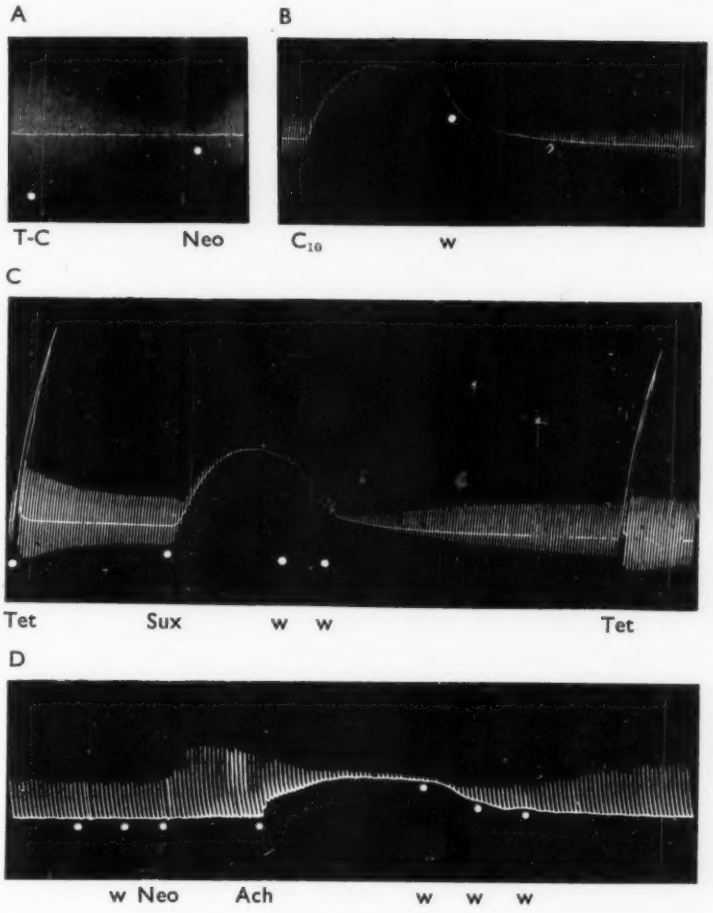

FIG. 2.—Contractions of isolated biventer cervicis muscle evoked by supramaximal nerve stimulation at 12/min. The tetani (Tet) in (C) were evoked by stimulation at 125/min., the maximum tetanic tension being 12 g. The contractions in (A), (B), and (C) were recorded with an "isometric" lever, and those in (D) were recorded using the mechano-electric transducer valve RCA 5734. (A) At T-C tubocurarine chloride was added to give a bath concentration of 1.4×10^{-5} M. At Neo, neostigmine bromide (3.3×10^{-6} M) was added. (B) At C_{10}, decamethonium iodide (3.4×10^{-8} M). (C) At Sux, succinylcholine dichloride (2.8×10^{-6} M) was added. (D) At Ach, acetylcholine chloride (1.3×10^{-6} M) was added before and after the addition of neostigmine bromide (6.6×10^{-6} M). At W, the bath was washed out.

muscle. The tendon and muscle were carefully removed together with the lower tendon which attaches the muscle to the supraspinous ligament. The anatomy of the chick has been described by Chamberlain (1943).

A loop which served to attach the preparation to the hook on the electrode assembly (Fig. 1) was tied around the lower tendon and the thread on the upper end of the muscle was passed through the electrode and attached either to a light semi-isometric lever writing on a smoked drum or to the lever of an RCA 5734 transducer valve suitably connected to a pen recorder. The electrode was lowered until it was in contact with the tendon surrounding the nerve. The organ bath (30 to 50 ml. capacity) contained Krebs-Henseleit (1932) solution which was maintained at a constant temperature between 37° and 40° and which was well stirred with a mixture containing 95% oxygen and 5% carbon dioxide. The preparation remained in good condition for several hours when stimulated supramaximally at a frequency of 12/min.

RESULTS

Fig. 2 (A) shows the effect of tubocurarine chloride (1.4×10^{-5} M in the bath) and the reversal of the neuromuscular block by neostigmine bromide (3.3×10^{-6} M). Fig. 2 (B) and (C) illustrates the contractures caused by decamethonium iodide (3.4×10^{-8} M) and succinylcholine dichloride (2.8×10^{-6} M). Fig. 2 (D) shows the effect of acetylcholine chloride (1.3×10^{-6} M) before and after the addition of neostigmine bromide (6.6×10^{-6} M).

DISCUSSION

The simplicity of the preparation should make it suitable for students' use.

We are grateful to Professor E. J. Zaimis and Dr. K. J. Child for showing us their techniques, and to Dr. R. B. Barlow for his help.

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Brit. J. Pharmacol. (1960), **15**, 412.

A NEW BIOLOGICAL METHOD FOR THE ASSAY OF DEPOLARIZING SUBSTANCES USING THE ISOLATED SEMISPINALIS MUSCLE OF THE CHICK

BY

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(RECEIVED MARCH 18, 1960)

The isolated semispinalis cervicis muscle of a 3 to 10 days old chick has been found to provide a very useful assay method for depolarizing substances. There are three main advantages of this preparation over that of the rectus abdominis of the frog: (*a*) quick relaxation without any artificial stretching of the muscle, (*b*) greater sensitivity to depolarizing drugs, and (*c*) no falling off in sensitivity for at least 2 hr.

A particular type of avian muscle is very sensitive to substances possessing an acetylcholine-like action at the neuro-muscular junction. For example, in adult fowls and chicks an intravenous injection of a minute dose of decamethonium or suxamethonium causes a rigid extension of the limbs and retraction of the head (Buttle and Zaimis, 1949). This is a peripheral effect, the muscles reacting to the depolarizing drugs with a true contracture (Zaimis, 1954, 1959). Because of this and in the hope of obtaining a sensitive and specific method for the quantitative determination of depolarizing drugs, the isolated leg and neck muscles of the chick were investigated. Of all the muscles, the semispinalis cervicis and the biventer cervicis muscles have been found to provide the best preparations as they are thin, very sensitive to depolarizing drugs, and easy to dissect.

The method was demonstrated for the first time to the Pharmacological Society in July, 1954, and a preliminary communication was given to the Biological Methods Group of the Society for Analytical Chemistry in June, 1954.

METHOD

Chicks usually obtained soon after hatching were housed at a temperature of 35 to 38° and kept on a diet of chick pellets and water.

After the chick had been anaesthetized with ether a longitudinal incision was made in the skin at the back of the neck from the base of the skull to the region of the thoracic vertebrae; the neck was then slightly flexed by means of a small pad of cotton wool. In the middle of the neck there are two pairs of thin muscles which arise from the second thoracic vertebra, run upwards side by side, and are inserted into the medial part of the occipital bone round the area of the nuchal crest. The superficial pair is formed by the two biventer cervicis, the deep one by the two semispinalis cervicis muscles. Either the biventer cervicis or the semispinalis can be used for this assay. Our preference, however, was given to the semispinalis because it could be dissected with the least possible damage. In order to expose the semispinalis, the biventer muscle of one side was lifted gently after cutting its proximal tendon (Fig. 1). The semispinalis muscle was then freed from the oblique muscles of the neck and a thread was tied round each end. Finally, the muscle was mounted as quickly as possible in a bath containing oxygenated Tyrode solution. In the present study a 5 ml. bath was used; the magnification was about 4 and the tension was 1 g.

The sensitivity of the muscle was at its best in chicks between 3 and 10 days old. In chicks less than 3 days old the muscle was very delicate and difficult to handle, whilst in chicks older than 10 days the sensitivity diminished and the differentiation between doses was less good. Differences in sex and breed did not appear to influence the response of the muscle. It was found that careful and gentle dissection was essential and that a muscle dissected from an anaesthetized chick was more sensitive than one from a recently killed animal.

Tyrode, Locke, de Jalon and Krebs solutions were tested. The sensitivity of the muscle was greatest in Tyrode solution at a temperature of 40 to 40.5°. While a temperature higher than 41° decreased the magnitude of the response without altering the time required for complete relaxation, a temperature below 39° affected both these factors; the response became less regular and the time required for complete relaxation increased markedly.

Pure oxygen and air were equally satisfactory for the oxygenation of the Tyrode solution provided that the air was finely dispersed.

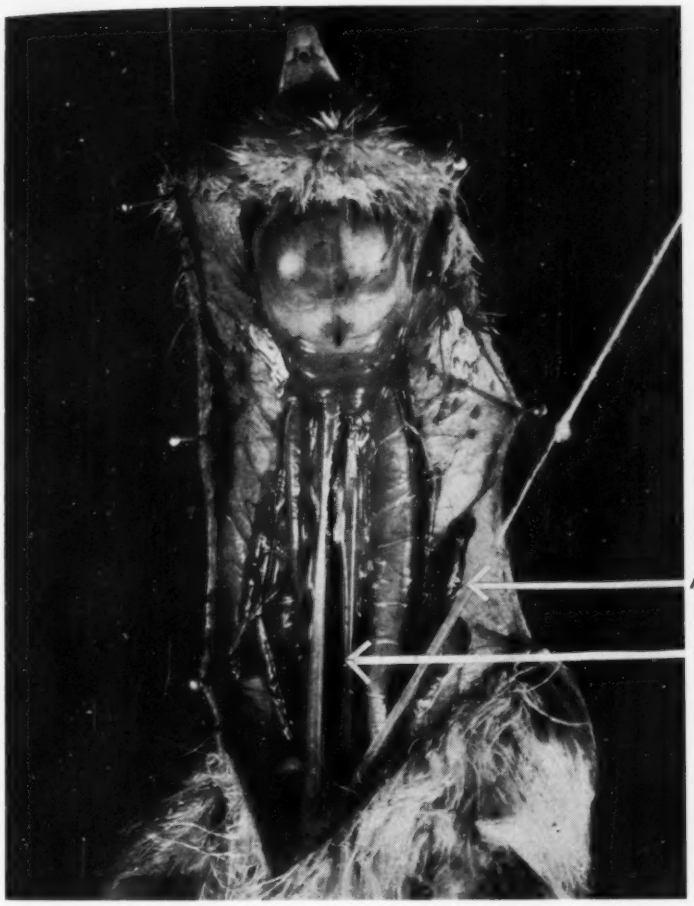

FIG. 1.—Chick; dissection of the back of neck to demonstrate (A) the biventer cervicis muscle retracted to show (B) the semispinalis cervicis muscle.

RESULTS

Substances Mimicking Acetylcholine

Decamethonium.—The semispinalis cervicis muscle responded with a sustained contracture to decamethonium and the magnitude of the response increased linearly with the logarithm of the dose (Fig. 2). The onset of the contracture was almost immediate with all but very small doses of the drug, when there was a latent period of 5 to 15 sec. The rate of development of the contracture was rapid at first but slowed down as the peak tension was approached; usually a plateau was reached in less than 1 min. The muscle remained in contracture until the bath was filled with fresh Tyrode. Relaxation was rapid and complete in less than 2 min. without any artificial stretching of the muscle. An interval of 4 min. between doses was almost always adequate.

Fig. 3 shows the result of an assay of a solution of decamethonium from which Tables I and II have been constructed. Two doses of "Standard" solution and two doses of "Test" solution were used in each assay, the doses being administered in the form of a Latin square. For the calculation of the ratio of potency and the analysis of variance of the results the method described by Schild (1942) was used with the difference that owing to the Latin square arrangement a further set of three degrees of freedom was set aside for "dose order." The value of M (log. ratio potency of unknown to standard) was estimated to be -0.0497 ± 0.003. The ratio of potency was therefore 0.892 and the potency of the test solution of decamethonium=0.892 μg./ml. The limits of error of the estimate=0.877–0.907 (P=0.05). The index of precision, $\lambda = s/b = 0.005$. In order to test the accuracy of the method the same solution of decamethonium of known strength was assayed on six fresh muscles, and the results and limits of error of the estimate of these assays are given in Table III. In each case there was a highly significant difference in activity between the two solutions and a highly significant regression between the smaller and larger doses making a quantitative estimate of activity possible. The low F value obtained for "groups" in every assay indicates that the sensitivity of the preparation did not change during the experiment.

Other Depolarizing Drugs.—Table IV shows the results obtained with suxamethonium and other substances possessing an acetylcholine-like action at the neuromuscular junction. The activity quoted for suxamethonium is the mean result of three estimations using decamethonium as the standard;

TABLE I

	Decamethonium μg./ml.	Dose ml.	Dose μg.	Responses mm.	Mean
A	1·0	0·60	0·600	32 32 32 32	32
B	1·0	0·45	0·450	14 15 16 15	15
C	0·9	0·60	0·540	25 26 24 25	25
D	0·9	0·45	0·405	9 9 8 9	8·75

TABLE II

THE ANALYSIS OF VARIANCE OF THE ASSAY RESULTS SHOWN IN TABLE I

The log. ratio of potency, M, = −0.0497 with limits of error ± 0.0074. The asterisked values of F are reciprocals.

Source of Variance	Sum of Squares	Degrees of Freedom	Variance	$F=V_1/V_2$	P
1. Groups	0·69	3	0·23	2·40*	Not significant
2. Standard and unknown ..	175·56	1	176	318	<0·001
3. Regression	1,105·62	1	1,106	2,000	<0·001
4. Dev. from parallelism ..	0·56	1	0·56	1·01	Not significant
5. Dose order	0·69	3	0·23	2·40*	,, ,,
6. Residual error	3·32	6	0·553		
Total	1,286·44	15			

the regression lines for these two substances did not differ significantly from parallel (in each case $P>0.1$).

The semispinalis cervicis muscle is very sensitive to depolarizing drugs; for example, concentrations of decamethonium and suxamethonium as low as 0.1 μg./ml. in the bathing fluid may be readily estimated. Furthermore, even less specific substances, such as carbachol and nicotine produced responses which could be easily measured.

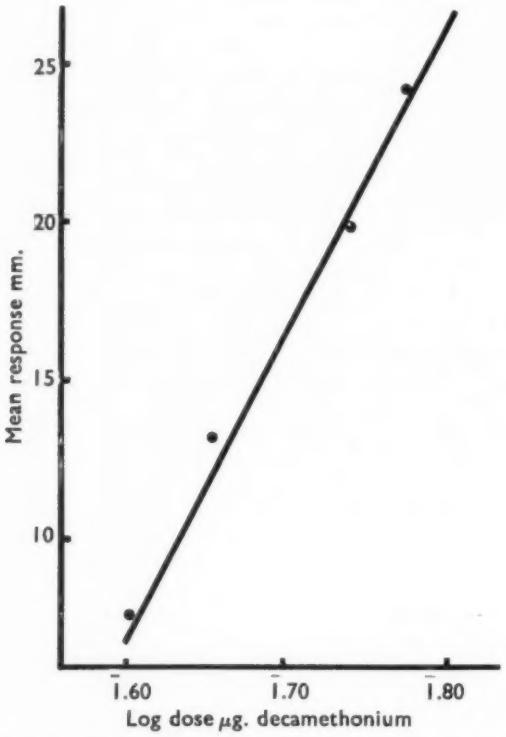

FIG. 2.—The log dose-response curve of decamethonium assayed on the isolated semispinalis cervicis muscle of the chick. S=0.59, b=93.89, λ=0.006.

TABLE III

ESTIMATION OF THE POTENCY OF A TEST SOLUTION OF DECAMETHONIUM CONTAINING 0.9 μG./ML. ON EACH OF SIX SEPARATE CHICK MUSCLE PREPARATIONS

Expt. No.	Estimated Potency μg./ml.	Limits of Error (P=0·05)	Dose Ratio	λ
1	0·908	0·878–0·939	5 : 4	0·009
2	0·892	0·877–0·907	4 : 3	0·005
3	0·889	0·864–0·915	4 : 3	0·009
4	0·896	0·868–0·924	4 : 3	0·011
5	0·889	0·865–0·913	5 : 4	0·009
6	0·885	0·859–0·910	6 : 5	0·009

TABLE IV

THE ACTIVITY OF VARIOUS DEPOLARIZING SUBSTANCES COMPARED WITH DECAMETHONIUM (=100)

All values refer to the bases.

Substance	Method of Assessment	Activity
Suxamethonium	Latin square assay	56–62 (mean=60)
Adipylcholine	Direct comparison	42
Acetylcholine	,, ,,	<1
Carbachol ..	,, ,,	<2
Nicotine ..	,, ,,	<2

Acetylcholine.—The response of the muscle to acetylcholine was unpredictable. In some preparations contracture was obtained with 5 μg. while in others 50 μg. produced no effect at all. However, in the presence of anticholinesterase drugs the sensitivity of the muscle increased and the responses became more predictable (Tyler, 1960).

Competitive Antagonists of Acetylcholine.—Competitive antagonists of acetylcholine antagonize the action of depolarizing drugs. Fig. 4 shows the effect of tubocurarine and hexamethonium; the response of the muscle to 0.5 μg. decamethonium may be reduced by approximately 50% by the previous administration of 2 μg. tubocurarine or of 100 μg. of hexamethonium. Fig. 5 shows the regression line relating log dose of tubocurarine and the percentage reduction of the response of the muscle to decamethonium.

In every experiment a potentiation of the response of the muscle to decamethonium was observed after tubocurarine had been washed out. Such an effect was not apparent following the administration of hexamethonium.

The Administration of Biological Fluids.—It was found that biological fluids containing depolarizing drugs could also be assayed provided they were approximately neutral. In acid ($pH < 6$) or alkaline ($pH > 8$) biological fluids the sensitivity of the muscle decreased very rapidly. In the alkaline fluids the decrease in sensitivity was sometimes preceded by a brief period of potentiation. Biological fluids tested included human blood and plasma, cat plasma, horse serum, and human urine. The administration of whole blood tended to reduce the rate of development of the contracture.

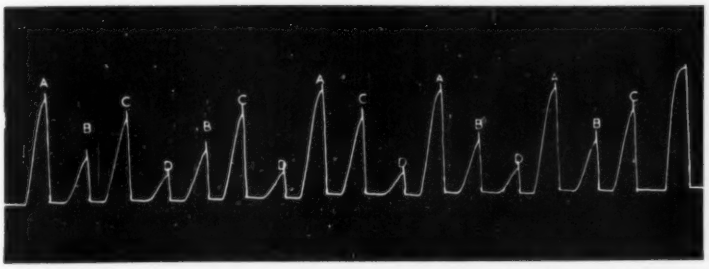

FIG. 3.—The estimation of potency of a decamethonium solution of unknown concentration on the isolated semispinalis cervicis preparation. A=0.60 ml. and B=0.45 ml. of a standard solution containing 1 μg./ml. of decamethonium. C=0.60 ml. and D=0.45 ml. of the test solution. Dose interval=3 min. 45 sec.

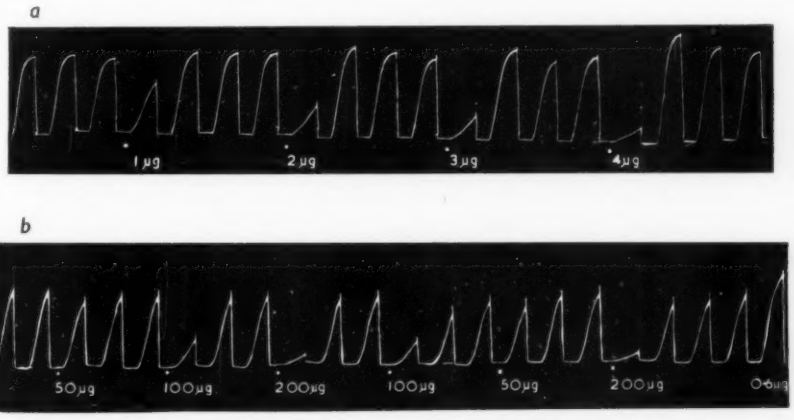

FIG. 4.—Contractions of the isolated semispinalis cervicis muscle produced by decamethonium 0·5 μg. Tubocurarine (1 to 4 μg.) (a), and hexamethonium (50 to 200 μg.) (b), were added to the bath 1 min. before the decamethonium at the points indicated. The last contraction was produced by 0·6 μg. decamethonium.

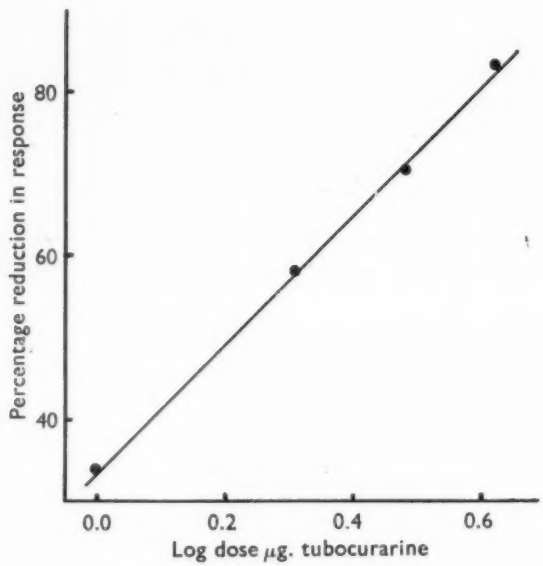

FIG. 5.—Log dose-response curve of tubocurarine assayed by its inhibitory effect on the decamethonium induced contractions of the isolated semispinalis cervicis muscle.

DISCUSSION

The semispinalis cervicis muscle of the chick, like the rectus abdominis muscle of the frog, responds by contracture to acetylcholine and to substances possessing an acetylcholine-like action at the neuromuscular junction. This muscle offers advantages over the rectus abdominis because (*a*) relaxation following contracture is much more rapid and occurs without any artificial stretching of the muscle; (*b*) the muscle is more sensitive to substances mimicking acetylcholine; and (*c*) there is no falling off in sensitivity for at least 2 hr.

The major part of this work was carried out at the Pharmacology Department of the School of Pharmacy, University of London. Grateful acknowledgement is made of a grant by the Medical Research Council (to E.Z.) for technical assistance.

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THE RELEASE OF ACETYLCHOLINE FROM MAMMALIAN MOTOR NERVE ENDINGS

BY

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The release of acetylcholine from rat and guinea-pig isolated diaphragm preparations stimulated through the phrenic nerve was optimal at 37° in Krebs solution with 5×10^{-6} neostigmine methylsulphate. The amount of acetylcholine released by a 20 min. tetanus was reduced by cooling. At frequencies of stimulation above 6/sec. the release was less than that predicted. This " failure " was unaffected by the addition of 1×10^{-6} choline. The acetylcholine release declined with continued stimulation at 25/sec. In the absence of nerve stimulation, there was a small continuous resting release of acetylcholine which seemed to originate in the muscle fibres. These results are discussed in the light of current electrophysiological knowledge of the quantal release of acetylcholine.

Nearly a quarter of a century has passed since Dale and his colleagues first showed that acetylcholine was released at the neuromuscular junction by motor nerve stimulation, and could be measured directly in the extracellular fluid if steps were taken to prevent its destruction by cholinesterase (Dale and Feldberg, 1934 ; Dale, Feldberg, and Vogt, 1936). Since that time it has been generally accepted that acetylcholine is the chemical mediator of nerve impulses at all neuromuscular junctions. Recently, Dale's observation has been confirmed and extended by others, in a perfused muscle preparation (Emmelin and MacIntosh, 1956) and in isolated diaphragm preparations (Burgen, Dickens and Zatman, 1949 ; Barnes and Duff, 1954 ; Brownlee, 1957 ; Brooks, 1954).

The present experiments are concerned with the factors which modify the amounts of the transmitter released from motor nerve endings ; and in particular with the effect of changes in the frequency and duration of stimulation, changes in temperature and the presence of choline on acetylcholine release.

METHOD

The Collection of Acetylcholine

The method was based on that of Burgen, Dickens, and Zatman (1949), and has been described in detail by Straughan (1959).

Female albino rats between 200 and 300 g. and female albino guinea-pigs of 200 g. were used. After decapitation, a half diaphragm with its attached phrenic nerve was put in a flat glass diaphragm bath containing between 2.5 and 3.0 ml. Krebs solution. The small volume has the advantage of increasing the acetylcholine concentration in the bath fluid. The upper intercostal margin of the diaphragm was connected by fine stainless wire to a semi-isometric spring lever.

To ensure complete inhibition of cholinesterase, the preparation was allowed to stand for 30 min. in 5×10^{-6} neostigmine methylsulphate before an experiment was begun. The temperature of the muscle was kept constant at $37° \pm 0.25°$ and the phrenic nerve was stimulated for 20 min. periods at a rate of 25/sec. with supramaximal rectangular pulses 0.03 millisecs. in duration, except where indicated.

Less acetylcholine was found to be released by the first than by subsequent periods of stimulation ; to offset this a " conditioning " 3 min. period of stimulation was applied to the preparation before each experiment. An intervening rest period of 10 min. was allowed between successive 20 min. periods of stimulation. Under these conditions the preparation could sustain up to eight successive 20 min. periods of stimulation before there was any appreciable fall in the amount of acetylcholine released.

In experiments to determine the recovery of acetylcholine at different concentrations of anticholinesterase, the initial equilibration was made at a low concentration of neostigmine, and the samples from two successive periods of stimulation collected. Subsequent samples were collected at gradually increasing concentrations of neostigmine.

When the effect of changing the frequency of stimulation on release was studied, the frequency of

stimulation in successive 20 min. periods was varied in a random order, so that there were two samples at each frequency. When the effect of duration of tetanus on release was studied, 20 min. periods of stimulation were alternated with 10 min. periods at the same frequency, so that there were three samples for each duration.

In experiments on the effect of temperature on acetylcholine release, the initial equilibration was always at 37°; samples were either collected at this temperature and at successively lower temperatures; or the muscle was cooled and samples collected at stages in the course of subsequent rewarming. Samples were withdrawn from the bath within a minute of the end of stimulation, acidified to about *p*H 4 with one drop of 5 N hydrochloric acid and frozen to −20° until required for assay.

The Estimation of Acetylcholine

The activity of small volumes of the bath fluid was matched against known concentrations of acetylcholine in Krebs solution containing neostigmine on the rat blood pressure preparation, as described by Straughan (1958; 1959). A male albino rat of 250 g. was anaesthetized with urethane 40 mg./100 g. and sodium pentobarbitone 3 mg./100 g. and allowed to cool to about 28° when a stable long-surviving preparation with a good blood pressure was obtained. Depressor artifacts which are normally a hazard of this preparation were almost completely avoided by carefully immobilizing the venous cannula after cutting the femoral nerves, and by ensuring that not more than 0.3 ml. of fluid was injected at one time. Sensitivity was highest in the winter months, when as little as 0.25 ng. acetylcholine base gave a measurable depressor response in the rat blood pressure.

The preparation also usefully discriminated between biologically active depressor substances; equi-depressor responses were seen with acetylcholine base 1 ng., choline base 10 μg., histamine 0.2 μg., adenosine triphosphate 1 mg., and potassium chloride 1 mg.

The identification of the depressor substance in the bath fluid as acetylcholine was based on the following observations.

1. The relative insensitivity of the assay preparation to other interfering biological substances.

2. The stability of the depressor substance in acid solution, and its destruction by boiling in an alkaline medium.

3. The absence of depressor activity in the bath fluid in the absence of adequate amounts of neostigmine (Table I).

4. The depressor activity of the bath fluid when present was abolished after atropine, and was enhanced by the previous administration of an anticholinesterase to the assay animal.

Krebs solution of the following composition was used (g./litre): NaCl 6.92, KCl 0.354, $CaCl_2$ 0.282, $NaHCO_3$ 2.1, KH_2PO_4 0.162, $MgSO_4,7H_2O$ 0.294, glucose 2.0.

TABLE I

ACETYLCHOLINE RECOVERY AND NEOSTIGMINE CONCENTRATION

Acetylcholine recovery in ng. base after 20 min. stimulation at 25/sec. in different concentrations of neostigmine methylsulphate. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Neostigmine Concentration					
	Absent	1×10^{-7}	5×10^{-7}	1×10^{-6}	5×10^{-6}	1×10^{-5}
1	0	4	—	—	58	—
2	—	1	15	—	33	—
3	—	—	19	37	71	—
4	—	—	—	22	51	51

A mixture of 95% oxygen and 5% carbon dioxide was bubbled through the solution. A correction was applied to the results for the small volume of fluid which remains in the diaphragm bath after draining and dilutes the subsequent addition of known volumes of fluid.

The resting release was measured in most experiments, but the results were not corrected for it, because of the larger errors involved.

RESULTS

The Effect of Anticholinesterase Concentration on the Recovery of Acetylcholine

The results from a number of experiments with the rat phrenic nerve diaphragm preparation showed that when neostigmine was omitted from the bath fluid no acetylcholine could be recovered

TABLE II

RESULTS FROM DIFFERENT DIAPHRAGMS SHOWING HOW THE RECOVERY OF ACETYLCHOLINE VARIES WITH THE CONCENTRATION OF NEOSTIGMINE

Means±S.E. are given. The numbers in parentheses indicate the number of diaphragms from which the means were derived. With each diaphragm two successive 20 min. periods of stimulation at 25/sec. were applied with the stated concentration of neostigmine.

Acetylcholine Recovery	Concentration of Neostigmine Methylsulphate
0 (4)	Absent
2±0·9 (3)	1×10^{-7}
14±1·9 (4)	5×10^{-7}
28±3·4 (6)	1×10^{-6}
52±3·6 (11)	5×10^{-6}
53±0·9 (3)	1×10^{-5}

after a 20 min. period of nerve stimulation at 25/sec. Little acetylcholine was recovered at 1×10^{-7} neostigmine methylsulphate, but at higher concentrations of neostigmine the recovery increased to become maximal at a 5×10^{-6} concentration of neostigmine salt. This is illustrated in Table I.

The collected results from these and other experiments (Table II) show that there was a statistically significant increase in acetylcholine recovery at each of the following concentrations of neostigmine methyl sulphate, 1×10^{-7}, 5×10^{-7}, 1×10^{-6}, and 5×10^{-6}. Thereafter further increase in the neostigmine concentration brought about no statistically significant increase in the recovery of acetylcholine after a period of stimulation. The possibility that neostigmine might increase the amount of transmitter released by each nerve impulse cannot be ignored. But Liley (1956a) has shown that 10^{-6} neostigmine did not alter the rate of the spontaneous quantal discharge of acetylcholine in the rat phrenic nerve diaphragm preparation.

The Effect of Changes in the Frequency of Stimulation on Acetylcholine Release in the Rat and Guinea-pig Diaphragms

The amount of acetylcholine released during a 20 min. collection from the rat diaphragm increased with the frequency of stimulation from 6/sec. to 25/sec. (Table III). But the amount of acetylcholine released did not increase in direct proportion to the number of stimuli applied, so that at 25/sec. it was less than half that expected (Fig. 1). This "failure" to release acetylcholine becomes even more marked at higher frequencies, and though the response of individual diaphragms was variable, in general the amount of acetylcholine released in each 20 min. period was steady within the range 25 to 100/sec. (Fig. 1).

TABLE III

ACETYLCHOLINE RELEASE AT DIFFERENT FREQUENCIES OF STIMULATION IN THE RAT AND GUINEA-PIG DIAPHRAGM PREPARATIONS

The acetylcholine release is given in ng. base per 20 min. stimulation. Each figure is the mean of two periods of stimulation applied at random to the particular diaphragm.

Expt.	Resting	Frequency of Stimulation				
		6/sec.	12/sec.	25/sec.	50/sec.	100/sec.
Rat						
1	5	33	41	59	—	—
2	10	34	43	61	—	—
3	7	—	44	56	38	50
4	10	—	51	69	69	55
5	—	—	25	29	48	55
Guinea-pig						
1	7	28	52	71	—	—
2	9	42	63	86	—	—
3	7	35	39	68	—	—
4	13	—	—	59	58	50
5	7	—	—	77	106	64
6	8	—	—	80	110	72

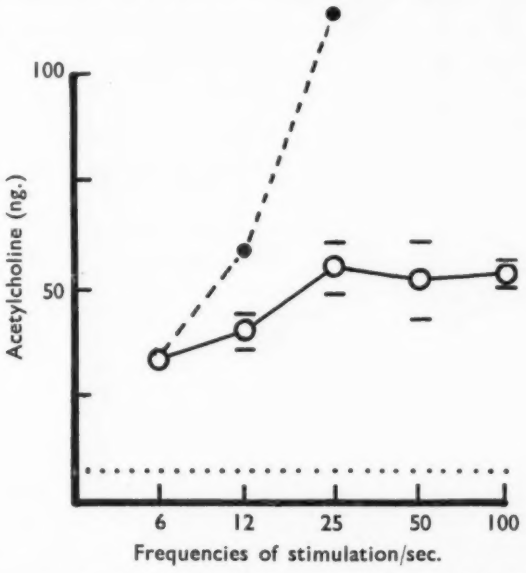

FIG. 1.—Variation in the amount of acetylcholine released from the isolated rat phrenic nerve diaphragm preparation at different rates of stimulation. Ordinate: ng. acetylcholine released per 20 min. period of stimulation in Krebs solution containing 5×10^{-6} neostigmine methylsulphate at 37°. Abscissa: rate of stimulation/sec. O——O : actual acetylcholine release. Each point is the mean ±S.E. from 2 to 6 diaphragms. ●– – –● : predicted acetylcholine release, calculated from the release at a stimulation rate of 6/sec. · · · · · : resting release of acetylcholine/20 min. collection period in the absence of stimulation.

In the guinea-pig diaphragm the amount of acetylcholine released per 20 min. period increased over the range 6 to 50 stimuli/sec. and thereafter declined (Table III and Fig. 2). "Failure" to release acetylcholine was not evident at 12/sec. and there was less "failure" at a frequency of 25/sec. than in the corresponding rat diaphragm, an observation which probably accounts for the relatively greater acetylcholine release in the guinea-pig diaphragm at this frequency.

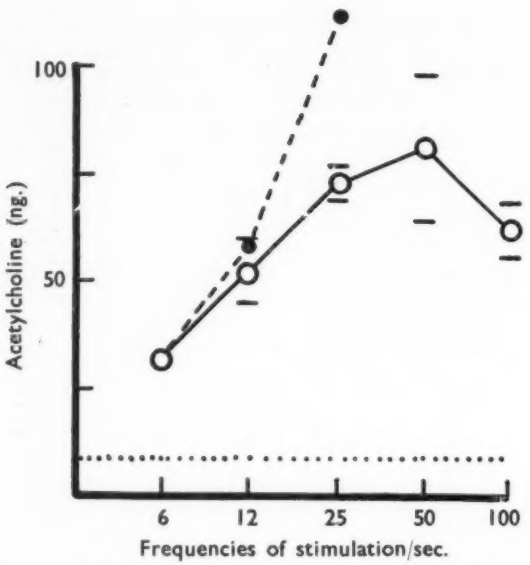

FIG. 2.—Variation in the amount of acetylcholine released from the guinea-pig phrenic nerve diaphragm preparation at different rates of stimulation. Ordinate: ng. acetylcholine base released per 20 min. period of stimulation in Krebs solution containing 5×10^{-6} neostigmine methylsulphate at 37°. Abscissa: rate of stimulation/sec. O——O: actual acetylcholine release. Each point is the mean ±S.E. from 2 to 6 diaphragms. ●– – –●: predicted acetylcholine release, calculated from the release at a stimulation rate of 6/sec. · · · · ·: resting release of acetylcholine 20 min. collection period in the absence of stimulation.

The Influence of the Duration of Stimulation on Acetylcholine Release in the Rat Diaphragm

The observed "failure" in the release of acetylcholine at some frequencies from the rat diaphragm might occur at the beginning of the period of stimulation, or might develop in intensity with continued stimulation. It is not practicable to measure the amount released each minute during a period of stimulation, so the acetylcholine released during 20 min. of stimulation at 25/sec. was compared with the acetylcholine released by 10 min. of continuous stimulation at the same frequency in each diaphragm.

In five experiments the release declined with continued stimulation during 20 min. more than 50% acetylcholine being released by 10 min. of stimulation (Table IV). In only one experiment was the acetylcholine release constant throughout 20 min. of stimulation, which suggested that uniform "failure" was present from the beginning of stimulation.

TABLE IV

DURATION OF STIMULATION AND ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Acetylcholine release in ng. base. Each figure is the mean of three alternate periods of stimulation at 25/sec. in that experiment.

Expt. No.	Duration of Stimulation		
	(a) 10 min.	(b) 20 min.	$\frac{2a}{b}$%
1	24	40	120
2	39	50	158
3	25	39	128
4	45	54	166
5	23	39	120
6	25	51	98

Perry (1953) has shown that with the cat's superior cervical ganglion perfused with saline there is a sharp decline in acetylcholine release after the first few minutes during prolonged stimulation, even at low frequencies. In this respect acetylcholine release from preganglionic and motor nerve endings is comparable, though the actual mechanism of failure is probably not identical.

The Influence of Temperature Changes on Acetylcholine Release in the Rat Diaphragm

Acetylcholine release from mammalian motor nerve endings has not been directly measured at different temperatures, though there have been indirect observations from electrophysiological studies by Boyd and Martin (1956), Liley (1956a) and Li (1958). The results (Fig. 3 and Table V)

TABLE V

THE EFFECT OF TEMPERATURE ON ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Release in ng. base per 20 min. at 25/sec. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Temperature			
	20°	30°	37°	40°
1	6	22	31	—
2	9	17	20	—
3	6	26	37	—
4	5	17	22	—
5	—	—	63	69
6	—	—	37	38
7	—	—	63	46
8	—	—	39	29

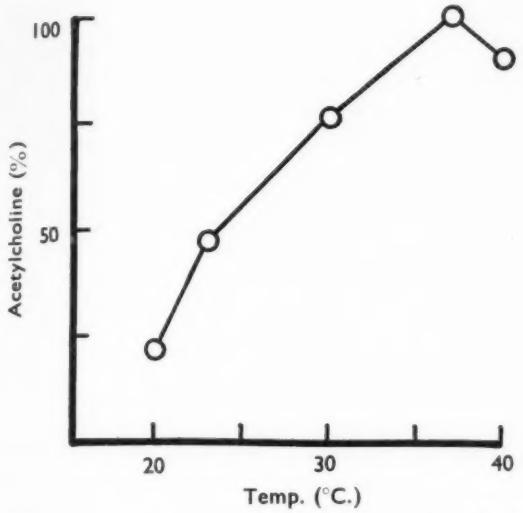

FIG. 3.—Variation in the amount of acetylcholine released from the rat phrenic nerve diaphragm preparation at different temperatures. 20 min. stimulation at 25/sec. in Krebs solution with 5×10^{-6} neostigmine methylsulphate. Ordinate: acetylcholine release as a percentage of the release at 37°. Abscissa: muscle temperature. O——O Each point is the mean release from 2 to 6 diaphragms.

showed that acetylcholine release varies directly with the temperature within the range of 20° to 37° whether acetylcholine release was studied after cooling or warming. The amount released at the two temperatures was significantly different at the 5% level of probability. The mean release of acetylcholine at 40° was less than at 37° (Table V), but this difference was not statistically significant at the 5% level of probability.

To provide a better basis for comparison between different diaphragms, the mean acetylcholine release in each experiment was plotted as a percentage of the release at 37°. The mean results from these different experiments are shown in Fig. 3.

The Effects of Choline on Acetylcholine Release in the Rat Diaphragm

The observation that the rat diaphragm preparation maintains a steady release of acetylcholine in response to repeated periods of stimulation suggests that, apart from the glucose in the Krebs solution, all the elements necessary for acetylcholine synthesis must be available in the preparation itself. Whether the absence of choline from the extracellular fluid imposed any restraint on the synthesis or release of acetylcholine was tested.

The results from four experiments (Table VI) showed that the presence of physiological amounts of choline (Bligh, 1952) brought about no significant increase in the amount of acetylcholine released by two 20 min. periods of stimulation at 25/sec.

TABLE VI

THE EFFECT OF CHOLINE ON ACETYLCHOLINE RELEASE IN THE RAT DIAPHRAGM

Acetylcholine release in ng. base/20 min./25/sec. Each figure is the mean of two successive periods of stimulation.

Expt. No.	Normal Krebs Solution	Krebs Solution + 1×10^{-6} Choline
1	46	60
2	51	41
3	37	32
4	38	42

The Resting Release of Acetylcholine from the Rat Diaphragm in the Absence of Nerve Stimulation

Brooks (1954) showed that there was a resting release of acetylcholine from the guinea-pig diaphragm preparation in the absence of nerve stimulation. A resting release has not yet been described in the isolated rat diaphragm preparation, though it was evident from the results of Burgen and others (1949) that a measurable release of acetylcholine still persisted in the isolated rat diaphragm preparation after paralysis with botulinum toxin; and Brooks (1956) has shown that any release of acetylcholine from motor nerve endings is abolished by this toxin.

The small amounts of acetylcholine involved in this resting release were measured in both rat and guinea-pig diaphragm by collecting samples over 20 min. collection periods at intervals of 1.5 hr. The mean resting release from 19 rat diaphragms was 7 ± 0.6 ng. (S.E.) acetylcholine base per 20 min.; and the mean resting release from 6 guinea-pig diaphragms (Table III) was 8.5 ± 0.6 ng. (S.E.) acetylcholine base. In both preparations the resting release appeared to be continuous and steady over many hours.

DISCUSSION

Acetylcholine Release and Frequency of Nerve Stimulation

If precautions are taken to prevent the destruction of acetylcholine, then variations in the amounts recovered after a period of nerve

stimulation under similar experimental conditions may be thought to have their origin in changes in the amounts set free at the nerve endings. This may occur in five principal ways.

1. By depletion of the stores of acetylcholine in the motor nerve terminals.

2. By altering the probability of quantal discharge in the terminal membrane. Liley (1956b) has shown that prolonged stimulation at high frequencies in the rat diaphragm has this effect, which probably accounts for the observation that less acetylcholine is released by the first period than by subsequent periods of tetanus, under identical conditions in the rat phrenic nerve diaphragm preparation.

3. By acting at some intermediate stage through which the nerve impulse co-ordinates the normal spontaneous discharge of quanta. Calcium and magnesium ions, particularly, appear to affect acetylcholine release by acting at this stage (Liley, 1956b ; Straughan, 1959).

4. By altering the level of depolarization produced by the nerve impulse in the terminals (Liley, 1956c). This is thought to be the mechanism by which small concentrations of procaine selectively reduce acetylcholine release (Straughan, 1959).

5. By altering the number of impulses invading the nerve terminals at particular synapses. Brooks (1954) suggested that high-frequency stimulation of motor nerves could cause a "blocking" reaction in the nerve terminals.

The most likely explanation for the observed "failure" to achieve the predicted release at frequencies above 6/sec. comes from the work of Krnjević and Miledi (1958b). They showed in the rat diaphragm preparation that, with stimulation of sufficient frequency and duration, there was an intermittent all-or-none failure of nerve impulse conduction, probably located in the narrow unmyelinated preterminal part of the nerve fibre. The observed decline in the amount of acetylcholine released during a 20 min. period of nerve stimulation is probably due to the fact that intermittent presynaptic failure is absent initially, and only appears and develops in degree with continued stimulation. The marked variation between different diaphragms in the amounts of acetylcholine which are released by nerve stimulation at rates above 25/sec. is perhaps due to the variation between different diaphragms in the time of onset, and degree of intermittent presynaptic failure.

It seems unlikely that there is any depletion of acetylcholine stores in the terminals during a tetanic period of stimulation even when there is complete neuromuscular block (Krnjević and Miledi, 1958b). The serial decline in the size of the end-plate potential in the curarized rat diaphragm with continued stimulation (Liley, 1956b) is due most likely to a gradual reduction in the acetylcholine sensitivity of the end plate (Krnjević and Miledi, 1958b) and an enhancement of the blocking activity of curare (Chou, 1947).

During a period of tetanus, therefore, there is a tendency for certain nerve impulses to be blocked intermittently, though when a nerve impulse does invade the terminals it will cause the normal amount of acetylcholine to be released from the terminals. This intermittent failure of conduction probably forestalls exhaustion of the terminal stores of acetylcholine (Castillo and Katz, 1956).

The Effects of Temperature on Acetylcholine Release

The most likely explanation for the observed decline in release with cooling over the range 37° to 28° is that cooling acts directly on the terminal membrane and reduces the probability of quantal discharge ; for the results are in fairly close agreement with the figures of Liley (1956a) and Li (1958), who measured the frequency of the spontaneous discharge in the rat diaphragm at these temperatures. These workers showed that with continued cooling to 20° there was a decrease and later an increase in the spontaneous discharge frequency, but Fig. 3 shows that the amount of acetylcholine released by a period of tetanus in fact decreased with continued cooling to 20°. This anomaly would be explained if, at and around room temperature, some further additive process were taking place, such as an increase in intermittent presynaptic failure at room temperature compared with 37° (as proposed by Krnjević, 1958), or possibly a reduced rate of acetylcholine resynthesis with cooling as suggested by Kostial and Vouk (1956) for the cat perfused superior cervical ganglion.

A decline in the amount of acetylcholine released with cooling over this range of temperature occurs in other nerve-muscle preparations. Boyd and Martin (1956) showed, in the curarized tenuissimus of the cat, that the amplitude of the end plate potential declined with cooling, while Bigland, Goetzee, Maclagan and Zaimis (1958) have stated that the blocking activity of curare was reduced by cooling. From their observation that the amplitude of the end plate potential in the cat tenuissimus blocked with magnesium increased on cooling, Boyd and Martin suggested that acetylcholine release was increased by cooling. But this last observation could be attributed more

easily to decreased curare-like and decreased depressant actions on acetylcholine release of magnesium ions at low temperatures.

Estimation of Acetylcholine Release per Synapse in the Rat Diaphragm

The figures in Table III show that about 2.5×10^{-8} g. of acetylcholine base is released from the rat diaphragm preparation by a 20 min. period of stimulation at 6/sec. after corrections are made for the resting release. Intermittent presynaptic failure which is thought to be precipitated by anoxia in the intramuscular portions of the motor nerve is minimal at this rate of stimulation (Krnjević and Miledi, 1958b, 1959); yet the post-synaptic changes observed in this preparation during stimulation at lower rates suggested that the deepest muscle fibres were anoxic (Creese, Hashish and Scholes, 1958). The use of neostigmine in the present experiments caused the rapid development of neuromuscular blockade, so by the end of the first minute of stimulation there was very little tension developed in the muscle. The absence of muscle activity during the greater part of stimulation thus makes it seem unlikely that severe anoxia occurred in the present experiments. If it is accepted that there is little or no "failure" in release at this frequency, then a single motor nerve volley released about 3.5×10^{-12} g. acetylcholine base. Accepting the calculations of Krnjević and Miledi (1958a) that there are 10,000 synapses in the rat diaphragm, it may be calculated that 3.5×10^{-16} g. of acetylcholine base will be released at a single synapse by a single maximal motor nerve volley in the rat diaphragm at 37° in Krebs solution. This is about three times as much as Acheson (1948) calculated was released at a nerve ending in the cat tongue. The difference may be a genuine species difference, or may arise from experimental errors.

Recent work by Liley (1956b) suggests that an individual quantum of acetylcholine in the rat diaphragm is between 1 and 2% of the amount normally released by a single nerve volley at the neuromuscular junction. From the figures presented above it would follow that each quantum would contain about 3.5×10^{-18} g. base or about 15,000 molecules of acetylcholine. This is higher than the revised estimate of 900 molecules per quantum for cat motor nerves given by MacIntosh (1959a).

It seems reasonable to suppose that most of the acetylcholine released at the nerve terminals is recovered under the conditions of the experiments, since the amounts needed to evoke substantial end plate potentials in the rat diaphragm (calculated from Krnjević and Miledi, 1958c) at 37° are only a hundred times greater than the release per synapse calculated from this present work. This discrepancy would probably disappear if it were possible to bring acetylcholine in as close proximity to the end plate as it is when naturally released.

Resting Release of Acetylcholine

The origin of the resting release was not clear; only a very small proportion of it could be accounted for by acetylcholine leakage from the cut end of the phrenic nerve, 0.6 ng. base/20 min. in one experiment; or by the spontaneous quantal discharge of acetylcholine estimated as 0.5 to 0.1 ng. base/20 min. by Straughan (1959). No great difference was apparent either between the resting release from the 7-day chronically denervated side of a diaphragm and the control acutely denervated side, in two preliminary experiments.

It would seem therefore that the resting release of acetylcholine originates in some non-nervous structure, perhaps the muscle fibres themselves. For MacIntosh (1959b) has pointed out that only half the preformed acetylcholine is lost from cat leg muscles after careful denervation.

It seems that the actual mechanisms of acetylcholine release from preganglionic and motor nerve terminals are not identical, and we must be cautious about extending results and knowledge from the one to the other. For instance, the release from motor nerve endings varies with the rate of stimulation in an almost identical manner to that observed in the cat superior cervical ganglion perfused with plasma (Birks and MacIntosh, 1957). But, in the ganglion perfused with saline the release declines sharply after the onset of stimulation so that there is little change in the amounts of acetylcholine released at different frequencies (Perry, 1953). It seems therefore that motor nerve endings maintain a high enough intracellular concentration of acetylcholine precursors to be independent of the organic composition of the extracellular fluid (apart from glucose), while the preganglionic nerve endings need choline and labile plasma factor for the optimal synthesis and release of acetylcholine (Birks and MacIntosh, 1957; MacIntosh, 1959a). It seems uncertain whether the mechanism of failure of acetylcholine release at high frequencies of stimulation in the ganglion perfused with plasma can be explained by intermittent presynaptic failure.

In contrast to motor nerve endings the acetylcholine release from the preganglionic nerve

endings of the cat superior cervical ganglion perfused with saline is not decreased by cooling (Kostial and Vouk, 1956). Thus it would appear that the acetylcholine release mechanism in preganglionic sympathetic nerve endings is not temperature-sensitive like the acetylcholine release mechanism in motor nerve endings. This implies that there is a fundamental difference between the terminal membranes of these two kinds of nerve endings. It will be of interest to see if acetylcholine release from preganglionic nerve endings is temperature-sensitive when the optimal conditions for synthesis and release are provided.

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THE INHIBITION BY MORPHINE OF THE ACTION OF SMOOTH MUSCLE STIMULANTS ON THE GUINEA-PIG INTESTINE

BY

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Morphine and related analgesics depress the responses of the isolated guinea-pig ileum to nervous stimulation and to drugs which act by stimulating the nervous structures of the intestinal wall. The present experiments show that these analgesics also depress the responses to smooth muscle stimulants which act directly on the smooth muscle fibres.

Morphine has a depressant action on the isolated intestine which is generally explained by an action on the nervous structures of the intestinal wall (Schaumann, Giovannini, and Jochum, 1952; Schaumann, 1955, 1956a; Kosterlitz and Robinson, 1955). Morphine also inhibits the action of drugs which cause contraction of the intestine by stimulation of these nervous structures (Schaumann, 1955; Kosterlitz and Robinson, 1955, 1958; Gaddum and Picarelli, 1957; Kosterlitz, Robinson, and Taylor, 1957). Part of the inhibition which morphine exerts through its action on the nervous structures may result from inhibition of the release of acetylcholine from post-ganglionic cholinergic fibres (Schaumann, 1956b, 1957; Paton, 1956, 1957). Kosterlitz and Robinson (1958) found that morphine strongly inhibits the contractions of the isolated guinea-pig ileum to nicotine, barium and 5-hydroxytryptamine, but has only a slight effect on the response to acetylcholine, carbachol and histamine. They concluded that the strong inhibition of the responses to nicotine, barium and 5-hydroxytryptamine was due to an action on the nervous elements innervating the muscle fibres, whereas the responses to acetylcholine and histamine were mainly on the muscle fibres and therefore resistant to the depressant action of morphine.

The present experiments show that morphine apparently has a non-specific depressant action on the smooth muscle fibres as well, since it depresses the action of drugs which stimulate these fibres directly.

METHODS

The guinea-pig ileum preparation was suspended in 15 ml. of Tyrode solution at 34°. Acetylcholine chloride, histamine acid phosphate (calculated as base) and carbaminoyl choline chloride (carbachol) were added at intervals of either 2 or 3 min. and left in contact with the tissue for 15 sec. Nicotine acid tartrate, barium chloride, potassium chloride, 5-hydroxytryptamine creatinine phosphate, bradykinin (Elliott, Horton and Lewis, 1960) and substance P (11 U./mg.) were added at intervals of either 4 or 5 min. and left in contact for 30 sec.

When the action of morphine, of related drugs and of hexamethonium was examined, their concentration was maintained by additions to the bath after each washing. The following drugs were used for this purpose: morphine sulphate, diamorphine hydrochloride (heroin), methadone hydrochloride, dihydromorphinone hydrochloride, codeine sulphate, hexamethonium bromide (vegolysen).

RESULTS

Morphine reduced the contractions of the guinea-pig ileum to smooth muscle contracting substances. The experiment of Fig. 1 shows the depression of acetylcholine responses by morphine 10^{-7} at *a*, 10^{-6} at *b*, and 10^{-8} at *c*. The threshold concentration of morphine for this effect is between 5×10^{-9} and 10^{-8}, but the effect depends on the concentration of morphine only within narrow limits. When a concentration of 10^{-7} is reached no further depression occurs even when the concentration of morphine is raised to 10^{-5}.

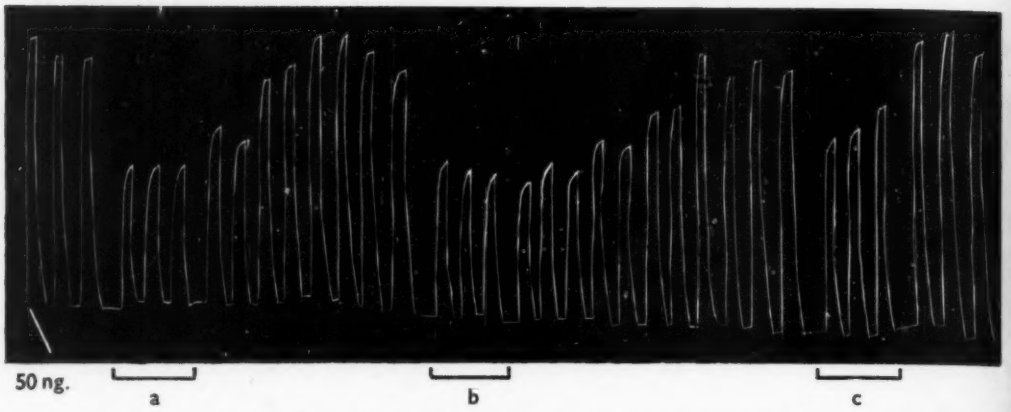

FIG. 1.—Responses of the guinea-pig ileum to acetylcholine 50 ng. alone and in the presence of morphine 10^{-7} g./ml. (*a*), 10^{-6} g./ml. (*b*), 10^{-5} g./ml. (*c*). Contractions every 3 min.; contact time 30 sec.

Usually the morphine has to be in contact with the tissue for 5 to 10 min. before it exerts its maximum effect, but some reduction in the responses is observed after morphine has been in contact for less than 1 min.

The duration of action of morphine after it is washed out will depend partly on the time it has been in contact and partly on the concentration used. With short contact times (of less than 2 min.) the time taken for the contractions to return to normal is proportional to the time of contact. Where the contact time is more than a few min. the recovery period depends on the concentration of morphine; at 10^{-8} recovery is almost immediate whereas at 10^{-5} the contractions do not return to normal for about 90 min.

Depression of the Action of Various Smooth Muscle Contracting Substances

The extent of the depressant action of morphine varies according to the drug used to contract the ileum, but in every experiment some depression was observed. The responses to acetylcholine and histamine were reduced about 20 to 40%. In the experiment of Fig. 2 morphine 2×10^{-7} reduced the response to acetylcholine 0.03 μg. to that previously given by 0.02 μg., and in the experiment of Fig. 3 it produced the same reduction with the responses to histamine. The experiment of Fig. 2 also illustrates that the depressant action of morphine still occurred when the ganglia were blocked by hexamethonium.

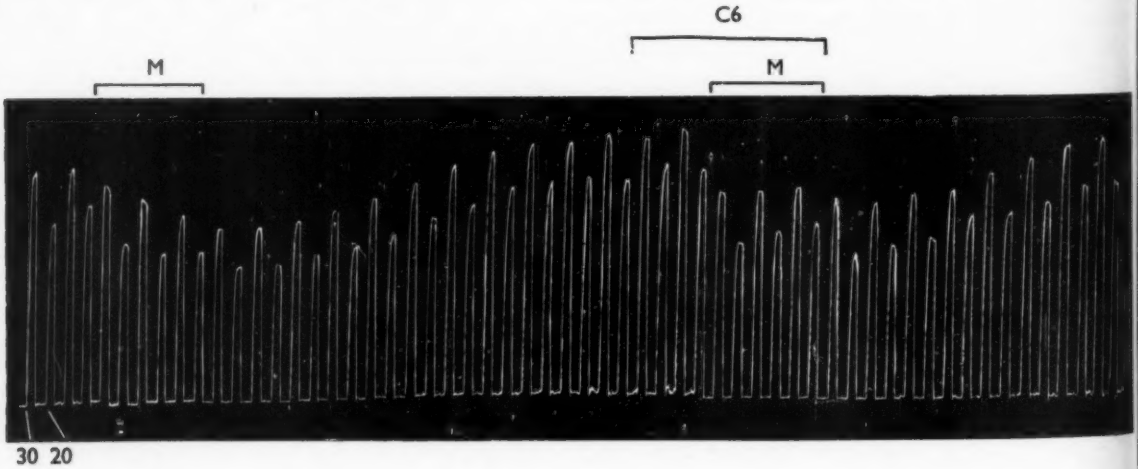

FIG. 2.—Responses of the guinea-pig ileum to acetylcholine 30 ng. and 20 ng. given alternately every 2 min. Contact time 15 sec. The bars at the top indicate the presence of morphine 2×10^{-7} g./ml. (M) or hexamethonium bromide 10^{-7} g./ml. (C6).

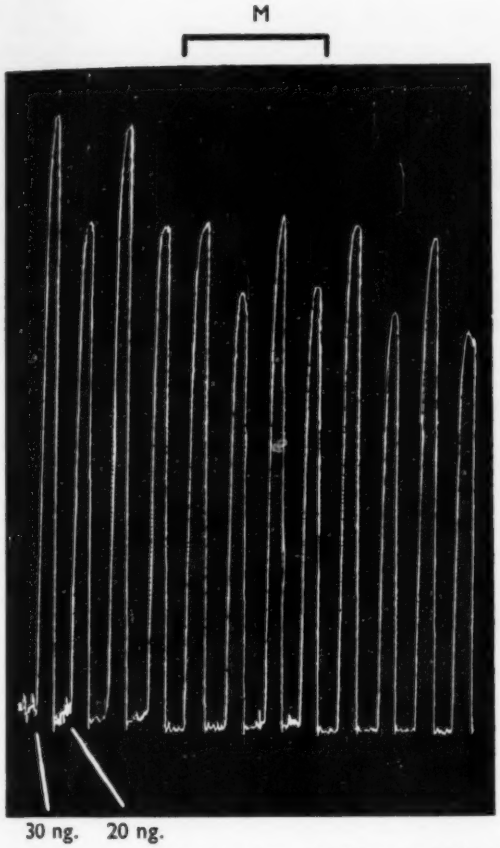

FIG. 3.—Responses of the guinea-pig ileum to histamine 30 ng. and 20 ng. given alternately every 2 min. Contact time 15 sec. The bar at the top indicates the presence of morphine 2×10^{-7} g./ml.

The experiment of Fig. 4 shows that the responses to both 5-hydroxytryptamine and nicotine were reduced to a much greater extent than those to histamine and acetylcholine, and the experiment of Fig. 5 shows that the responses to barium chloride were reduced much more than those to potassium chloride.

Morphine depressed the action of the various drugs to the same extent throughout their whole dose-response curves. In Fig. 6 the responses to acetylcholine are plotted on a graph to give the dose-response curve of acetylcholine alone and in the presence of morphine. The distance between the two curves gives the ratio of the doses of a substance causing equal contractions in the absence and in the presence of the inhibitor. This figure, which is a convenient measure of inhibition, has been called the dose ratio (Gaddum and Picarelli, 1957). In the experiment of Fig. 6 this figure was 1.7. The dose ratios for the other substances tested are shown in Table I, together with the standard errors. With the drugs in column 1, the dose ratios varied only between 1.42 and 1.82 and the variability of the morphine depression was relatively small, as shown by the standard errors. However, with nicotine, barium chloride and 5-hydroxytryptamine, the drugs in column 2, the dose ratios were not only greater but also more variable as shown by the large standard errors.

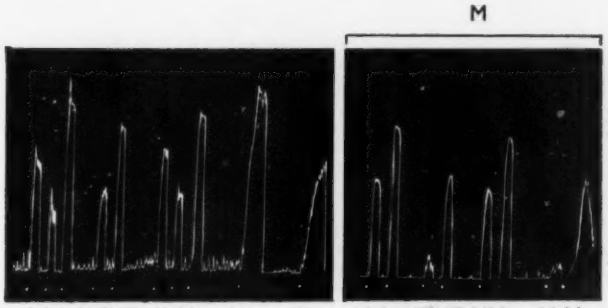

FIG. 4.—Response of the guinea-pig ileum to acetylcholine (A), 5-hydroxytryptamine (HT), histamine (H), and nicotine (N) alone and in the presence of morphine 2×10^{-7} g./ml. (M). Doses are given in μg. added to the organ bath.

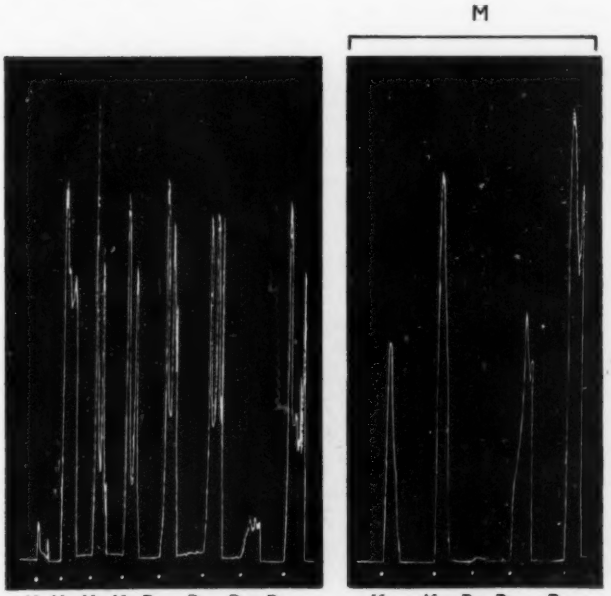

FIG. 5.—Responses of the guinea-pig ileum to potassium chloride (K) and barium chloride (Ba) alone and in the presence of morphine 2×10^{-7} g./ml. (M). Doses are given in mg. added to the organ bath.

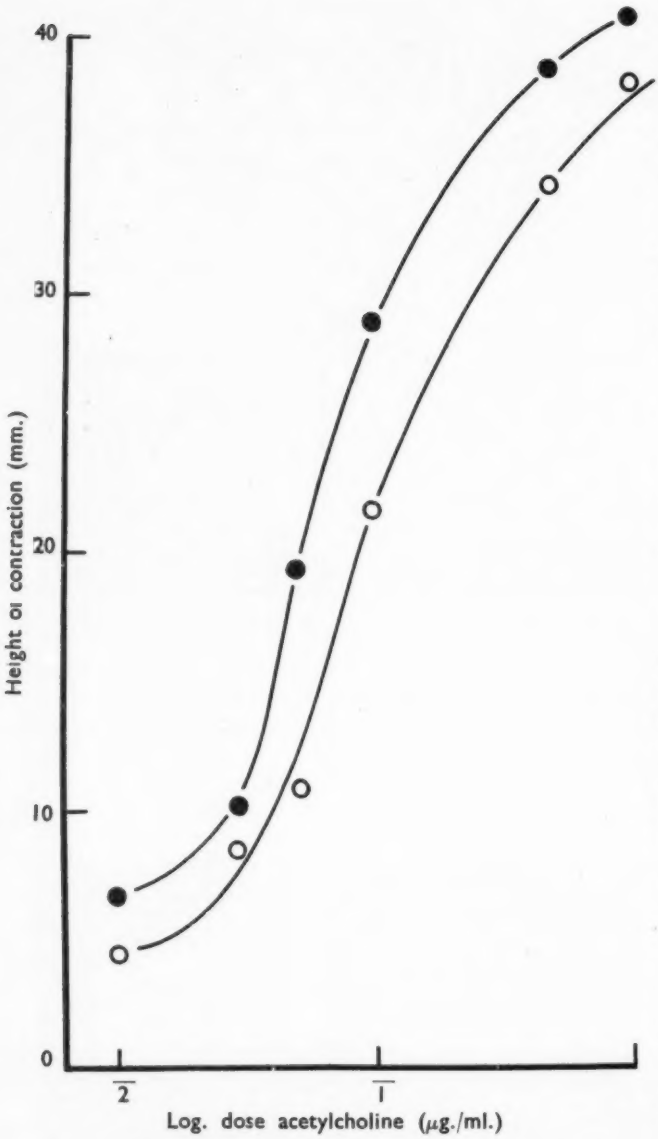

FIG. 6.—Dose-response curve of acetylcholine on the guinea-pig ileum, alone (closed circles) and in the presence of morphine 2×10^{-7} g./ml. (open circles). The points are plotted from the results of the experiment of Fig. 6.

The results of Table I thus show that drugs which contract the guinea-pig ileum primarily by a direct action on the muscle fibres have lower dose ratios than those which act on the nervous structures in the intestinal wall like nicotine, or on the nervous and muscle structures like barium chloride.

Drugs Related to Morphine

The inhibition of the action of smooth muscle stimulants on the guinea-pig ileum is also shown by analgesic drugs related to morphine, such as methadone, heroin, codeine, and dihydromorphinone. Usually methadone and heroin caused depressions at a slightly lower threshold concentration than morphine, whereas with codeine the threshold was higher than that for morphine. The experiment of Fig. 7 shows the depression of responses to carbachol and substance P by morphine 10^{-7} and by codeine 5×10^{-6}.

Adaptation to Morphine

In some experiments the guinea-pig intestine became tolerant to the action of morphine; after several doses, morphine no longer depressed the action of smooth muscle stimulants. This development of tolerance was more frequently obtained with large doses of morphine, but even under this condition it did not occur in all preparations.

When an intestine had become tolerant to morphine and larger doses were then applied, they did not decrease the responses to smooth muscle stimulants but increased them. Usually in these instances another peculiar effect was seen; when the morphine was withdrawn from the organ bath the responses were depressed, but could be restored on renewed addition of morphine to the bath, so that the intestine seemed to be dependent on the presence of morphine for its normal responsiveness. Such an experiment is illustrated in Fig. 8. Responses to barium chloride were at first inhibited by morphine 10^{-6}. Later when morphine was continuously in contact with the tissue the responses gradually recovered, and when the dose of morphine was raised to 5×10^{-6} and then to 10^{-5} the responses increased. On withdrawal of morphine the responses were depressed but were restored again when morphine was added during one contraction only. When the morphine was withdrawn again the responses were again depressed. In several intestinal strips tolerance with dependence was obtained and in some neither dependence nor tolerance could be produced.

Spontaneous Activity

When spontaneous activity of the intestine was present it was depressed by the concentrations of morphine which reduced the action of smooth muscle stimulants. This effect is seen in Figs. 4, 5 and 7. However, when the intestine became tolerant, morphine sometimes increased spontaneous movement, and on withdrawal of

TABLE I

DOSE RATIOS

The ratio of the doses of various smooth muscle stimulating drugs giving equal response before and after morphine 2×10^{-7} g./ml.

	Mean	No. of Expts.	Standard Error of Mean		Mean	No. of Expts.	Standard Error of Mean
Acetylcholine ..	1·61	10	0·086	Nicotine	3·00	6	0·465
Histamine	1·77	6	0·093	Barium chloride ..	4·17	6	0·857
Plasma kinin ..	1·75	4	0·171	5-Hydroxytryptamine	3·20	7	0·485
Substance P ..	1·42	5	0·120				
Carbachol	1·60	4	0·131				
Potassium chloride	1·82	4	0·075				

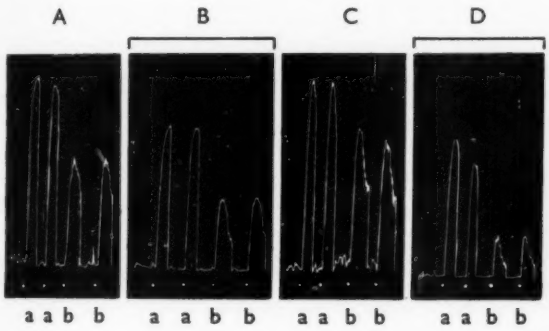

FIG. 7.—Responses of the guinea-pig ileum to carbachol 0·1 μg. (*a*) and substance P (*b*), alone (A), in the presence of morphine 10^{-7} (B), alone after 90 min. recovery (C) and in the presence of codeine 5×10^{-6} (D). Time interval 3 min.; contact time 15 sec. for carbachol, 30 sec. for substance P.

morphine during the phase of dependence, the contractions produced by barium chloride or acetylcholine were characterized by rhythmic activity as shown in Fig. 8.

DISCUSSION

It is evident from the present experiments that when we consider the action of morphine on the guinea-pig intestine we have to assume an action not only on the nervous structures of the intestinal wall, but also on the smooth muscle fibres themselves.

The finding that the contractions caused by substances which primarily stimulate smooth muscle directly are depressed to a smaller extent than those caused by substances which act on nervous structures is understandable because

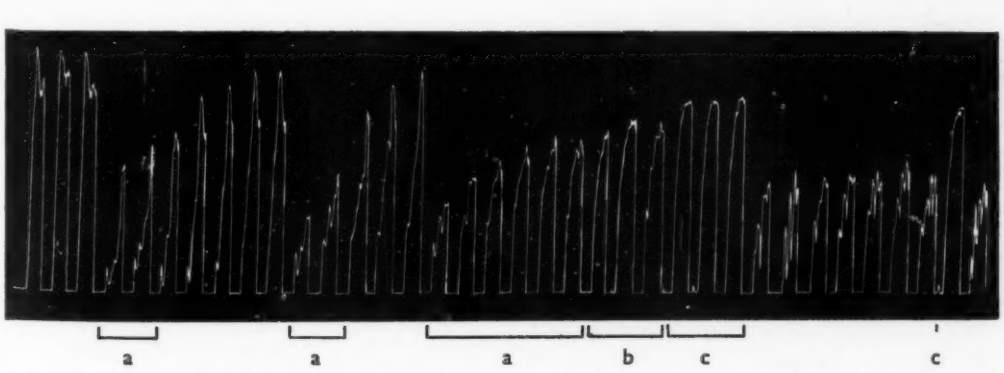

FIG. 8.—Responses of the guinea-pig ileum to barium chloride 3 mg. given every 5 min. Contact time 45 sec. The period marked by bar (*a*) indicates the presence of morphine 10^{-6}, (*b*) morphine 5×10^{-6}, (*c*) morphine 10^{-5}.

morphine has a two-fold depressing effect on the responses to substances which act on the nervous structures. In the case of histamine, for instance, morphine has only one site of action—depression of the muscle fibre—whereas with nicotine it has two sites of action—reduction of acetylcholine released from postganglionic cholinergic fibres and depression of the action of the released acetylcholine on the muscle fibre. In the case of barium chloride morphine probably has a three-fold depressing action; it reduces the release of acetylcholine, and it inhibits the action of the released acetylcholine, and it depresses the direct smooth muscle stimulating effect of barium chloride. The wide range of dose ratios (as shown by standard error) for drugs which act on nervous structure or nervous and muscle structures is consistent with the view that morphine is acting at more than one site and therefore subject to greater variability.

It is established that acetylcholine, histamine, carbachol and potassium chloride contract the guinea-pig ileum mainly by direct stimulation of the muscle and that nicotine acts wholly in the ganglia whilst barium chloride acts both on the nervous structures as well as on smooth muscle (Feldberg, 1951; Ambache and Lessin, 1955). There is also evidence that 5-hydroxytryptamine causes contraction of the guinea-pig ileum by acting on a nervous structure, although this action is different from that of nicotine and barium chloride in being insensitive to the action of hexamethonium or large doses of nicotine (Rocha e Silva, Valle and Picarelli, 1953; Robertson, 1953). Gaddum and Picarelli (1957) have postulated the existence of two kinds of tryptamine receptors, one of which they call morphine, or M receptors which can be blocked with morphine. However, the results of the present experiments do not support the idea of such a specific action of morphine. Therefore the name morphine or M receptor would appear to be misleading, although the concept of tryptamine derivatives having two sites of action would not be invalidated.

The possibility must be considered that the cholinergic nerves in the intestine like cholinergic nerves elsewhere are continually releasing quanta of acetylcholine while "at rest." Such releases, themselves producing responses below threshold for contraction, could nevertheless sum with added acetylcholine or other smooth muscle contracting substances. Morphine, by abolishing the release of such subthreshold quanta, would increase by a slight amount the concentration of added smooth muscle stimulant required to produce a given contraction. An explanation of this kind would support the view that morphine has an entirely neuronal action. However, the dose-response curve of a smooth muscle stimulating substance such as acetylcholine is shifted to a parallel position on the right by morphine. But if morphine depressed only the quanta of acetylcholine released "at rest," then the shape of the dose-response curve would be altered to indicate a greater percentage depression with small doses than with large doses.

When discussing the action of morphine in reducing the release of acetylcholine from cholinergic nerve fibres, Schaumann (1957) concluded that morphine inhibits the excitatory processes which release acetylcholine from nerve endings. Recently Schaumann (1958) has suggested that analgesics like morphine might depress the excitability of the intestine by liberating noradrenaline within the intestinal wall. According to his results, noradrenaline inhibits the release as well as the action of adrenaline. The inhibition observed in the present experiments was similar in degree to the reduction in acetylcholine release found by Schaumann. There is also a remarkable similarity to the morphine depression described by Paton in his experiments with co-axial stimulation. The threshold concentration is the same, and above a concentration of about 2×10^{-7} no further depression can be effected for the action on smooth muscle or for the release of acetylcholine. Onset and duration of both activities are similar, and both are effected not only by morphine but also by analgesic drugs related to morphine. In both cases it was sometimes possible to produce morphine tolerance and occasionally morphine dependence where, when morphine was withdrawn, the responses of the intestine were reduced and could be restored by fresh additions of morphine.

Thus morphine appears to act on the nervous structures of the intestine in much the same way as on smooth muscle and the underlying mechanism may well be an inhibition of a metabolic process common to both nervous and muscle structures.

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MAXIMAL INHIBITION OF CHOLINESTERASE IN THE CENTRAL NERVOUS SYSTEM

BY

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In experiments on mice treated with pralidoxime iodide (pyridine-2-aldoxime methiodide; PAM) and atropine, the cholinesterase activity in the brain was assayed after poisoning with very high doses of organophosphorous anticholinesterases. Acetylcholine was added to the buffer solution in which the brains were homogenized. This precaution reduced the combination between free inhibitor present in the tissue and active enzyme, and the cholinesterase activity found was below 0·5% of controls. When the experimental data were corrected for spontaneous reactivation *in vitro* during incubation, the calculated activities *in vivo* were even less. It is concluded that mice can survive complete inactivation of the cholinesterase in the central nervous system, if enough atropine is given to protect the animals against the toxic effects of the accumulating acetylcholine.

Death after poisoning with anticholinesterases which penetrate into the central nervous system is due to both central and peripheral inhibition of cholinesterase. Pralidoxime iodide (pyridine-2-aldoxime methiodide; PAM) effectively protects the peripheral cholinesterase or reactivates it after inhibition by organophosphates (Kewitz and Nachmansohn, 1957), whereas atropine protects against the actions of the accumulating acetylcholine in the central nervous system. In combination with pralidoxime iodide, atropine increases the LD50 about 10 times (Askew, 1957; Hobbiger, 1957; Kewitz, Wilson, and Nachmansohn, 1956; Wills, Kunkel, Brown, and Groblewski, 1957). The question arises whether the brains of mice surviving excessive doses of organophosphates under these conditions contain any active cholinesterase.

Several experimental difficulties must be overcome in order to answer this question:

1. After high doses of organophosphates, the brain contains large amounts of free inhibitor which must be prevented from blocking the remains of active enzyme during and after homogenization. This was done by adding acetylcholine to the buffer before homogenization (Schaumann, 1960a).

2. After poisoning with diethyl phosphates, there is spontaneous reactivation *in vitro* and the experimental values must be corrected accordingly (Schaumann, 1960a).

3. Labile organophosphates like paraoxon are destroyed enzymically in the body so quickly that reactivation of inhibited cholinesterase *in vivo* very soon prevails over inactivation by free inhibitor (Schaumann and Schiller, 1960a). Therefore, only anticholinesterases with a long half-life were used in the present experiments.

METHODS

Male white mice received 50 mg./kg. pralidoxime iodide + 10 mg./kg. atropine intraperitoneally and at the same time one of the following anticholinesterases subcutaneously: diethyl-dimethylaminothioethyl-phosphate acid oxalate (217-AO), parathion (*OO* - diethyl - *O* - *p* - nitrophenyl - phosphorothionate; E 605), or dyflos. The animals were decapitated 1 or 2 hr. after injection. The brains were dissected at once, weighed and homogenized with 1.9 ml. buffer solution per 100 mg. of brain. The buffer contained 7.5 to 10 mM. acetylcholine, 0.03 M magnesium chloride, and 0.03 M sodium bicarbonate. Two ml. of the homogenate was pipetted into Warburg vessels and at once incubated at 37°. The manometers were gassed with 95% nitogen + 5% carbon dioxide, and the production of carbon dioxide was measured from 30 to 90 and from 90 to 150 min. after inserting the manometers. The concentration of acetylcholine in the homogenates was determined colorimetrically according to the method of Hestrin (1949) at the beginning of the incubation and after 3 hr. The carbon dioxide production from brain homogenates to which an excessive amount of an anticholinesterase had been added was subtracted as a blank from the manometric values. The colorimetric readings were corrected for the spontaneous hydrolysis of acetylcholine during incubation. Cholinesterase activity

was calculated from the production of carbon dioxide (manometric determination) or the consumption of acetylcholine (colorimetric determination). The homogenate of a normal mouse brain diluted 1:20 served as a control. All determinations were done in duplicate.

RESULTS

Determination of Cholinesterase Activity in vitro

The manometric determination of cholinesterase activity during two successive hours shows a measurable increase in activity after poisoning with 217-AO and parathion (Table Ia). The rate of reactivation is practically linear during this time, and the readings taken between 30 and 90 min. after the beginning of the incubation therefore represent the activity after 60 min. (a_{60}); those of the second period the activity after 120 min. (a_{120}).

The reactivation follows the equation:

$$\ln \frac{100-a_o}{100-a_t} = K_r \cdot t \ldots\ldots (1)$$

a_o, a_t = % activity after 0 and t min. of incubation; t = duration of incubation in min., K_r = first order rate constant for the reactivation.

When the average activities for a_{60} and a_{120} from Table I are inserted together with t = 60 in equation (1), K_r can be calculated. K_r was lower after 60 mg./kg. of parathion—i.e., the rate of reactivation was slower than after 217-AO. Apparently, the high concentrations of free inhibitor found after parathion (Schaumann and Schiller, 1960b) reduced the speed of reactivation in spite of the addition of acetylcholine.

The cholinesterase inhibited by dyflos is not reactivated (Hobbiger, 1957; Davison, 1953). It was, therefore, sufficient to determine the enzyme activity once. The activities found are valid for the whole incubation period (Table II).

The colorimetric determinations give an average of the cholinesterase activity during the whole incubation period. The rate of reactivation being practically linear, the figures can be compared with the activities measured manometrically at the middle of the incubation period, i.e., after 90 min. (a_{90}). It follows from equation (1) that a_{90} is the geometric mean between $100-a_{60}$ and $100-a_{120}$. As shown in Tables I and II, there is a good agreement between the results obtained with both methods. In spite of low activities, the manometric determination gave just as dependable results as the colorimetric determination under the present experimental conditions.

Calculation of Cholinesterase Activity in vivo

No more than 3 min. elapsed between killing the animals and inserting the manometers. Therefore, the activity at the beginning of the incubation period, a_o, represents the cholinesterase activity *in vivo*. For 217-AO and parathion, a_o can be calculated by inserting into equation (1) t = 90, a_{90} from Table I for a_t and the respective values for K_r. No correction is necessary with dyflos. The results are summarized in Table III. When the mice were killed 1 hr. after the injection of 10 mg./kg. of 217-AO, a cholinesterase activity of 3.5–3.9% was found after 90 min. of incubation. From these values an activity *in vivo* of 0.04 to 0.3% of normal can be

TABLE I

MEASURED CHOLINESTERASE ACTIVITY OF BRAIN

The mice were killed (a) 1 hr. after 10 mg./kg. 217–AO subcutaneously, and (b) 2 hr. after 60 mg./kg. parathion subcutaneously; in both substances 50 mg./kg. pralidoxine iodide + 10 mg./kg. atropine was also given intraperitoneally. All values are % of control activity.

(a)

Exp. No.	Cholinesterase Activity			
	Manometrically			Colorimetrically
	a_{60}	a_{120}	a_{90}	a_{90}
3	0·9	2·6	1·75	2·10
	2·6	5·3	3·95	3·60
	2·6	5·4	4·00	3·60
	2·2	4·5	3·35	3·30
4	3·1	5·4	4·25	3·70
	4·2	6·1	5·15	4·20
	3·2	5·8	4·50	4·00
	2·8	5·0	3·90	3·50
Mean:	2·7	5·0	3·86 ±0·35	3·50 ±0·22
	$K_r = 0{\cdot}0004$			

(b)

5	1·6	3·4	2·50	2·6
	1·7	3·3	2·50	2·8
	2·1	4·0	3·05	3·1
	2·2	4·4	3·30	3·4
6	1·5	3·1	2·30	2·1
	2·5	4·2	3·35	2·9
	2·0	3·2	2·60	2·3
Mean:	1·94	3·67	2·80 ±0·16	2·74 ±0·22
	$K_r = 0{\cdot}000296$			

TABLE II

MEASURED CHOLINESTERASE ACTIVITY OF BRAIN

The mice were killed 1 hr. after injection of 80 mg./kg. dyflos subcutaneously and 50 mg./kg. pralidoxime iodide + 10 mg./kg. atropine intraperitoneally. All values are % of control activity.

Exp. No.	Cholinesterase Activity	
	Manometrically	Colorimetrically
1	0·45	0·45
	0·40	0·50
	0·20	0·45
	0·45	0·55
2	0·75	0·85
	0·70	0·95
	0·53	0·55
	0·55	0·50
Mean:	0·50 ±0·06	0·60 ±0·07

TABLE III

CALCULATED CHOLINESTERASE ACTIVITY OF BRAIN *IN VIVO*

mmt.=manometric; col.=colorimetric determination. All values are % of control activity.

	Cholinesterase Activity after Subcutaneous Injection of:		
	217-AO 10 mg./kg.	Parathion 60 mg./kg.	Dyflos 80 mg./kg.
In vitro (a_{90})			
mmt. ..	3·86	2·80	0·50
col. ..	3·50	2·74	0·60
In vivo (a_0)			
mmt. ..	0·33	0·17	0·50
col. ..	0·04	0·11	0·60

calculated. Two hr. after 60 mg./kg. of parathion the figures were even lower and from the activities found after 90 min. incubation and the rate constant of reactivation given in Table Ib, a cholinesterase activity *in vivo* of 0.1 to 0.2% of normal can be calculated. After injection of 40 mg./kg. of dyflos, Kewitz and Nachmansohn (1957) found 1.4% cholinesterase activity in the brain. In the present experiments, 80 mg./kg. was tolerated, and the activity was reduced to 0.5 to 0.6% of normal.

DISCUSSION

Using a histochemical method, McIsaac and Koelle (1959) found that physiologically liberated acetylcholine is hydrolysed only by the so-called functional cholinesterase on the cell surface and not by the reserve cholinesterase within the cells. Pharmacologically equipotent doses of two organophosphates inhibited the functional enzyme to the same degree, but only one of them penetrated into the cells; the other one left the reserve cholinesterase practically intact. In consequence, the tissue homogenates showed a considerably higher cholinesterase activity after the latter compound.

The cholinesterase activity in the brains of mice poisoned with equitoxic doses of different organophosphates decreases inversely with the stability of the respective compound in the body. In other words, the longer the half-life of a substance in the brain, the lower are the cholinesterase values found after a lethal dose (Schaumann, 1960b). This is confirmed by the present results (Table III). The free inhibitor found in mouse brain after injection of dyflos has a half-life of about 15 min. after 217-AO of about 40 min., whereas after parathion there is no measurable decrease in free inhibitor during 10 hr. (Schaumann and Schiller, 1960b). The mice were still alive 2 hr. after the injection of 60 mg./kg. of parathion, but they died 1 to 3 hr. later, when the therapeutic effects of pralidoxime iodide and atropine had worn off. A stable inhibitor will have more time to penetrate into the cell than one which is quickly metabolized. It is therefore most likely that the activities remaining after dyflos and 217-AO are due to inner or reserve cholinesterase.

Even if one assumes that the minute enzyme activities *in vitro* were due to external, functional cholinesterase, they do not constitute the absolute minimum necessary for the maintenance of life. In spite of pralidoxime iodide treatment, the animals showed severe muscular fasciculations and fibrillations, symptoms characteristic for peripheral inactivation of cholinesterase. It has previously been shown that in mice pretreated with pralidoxime iodide and atropine, the injection of a lethal dose of 217-AO reduced the cholinesterase activity in the diaphragm to the critical value for a lethal neuromuscular block in spite of the reactivator. When artificial respiration was given in addition to pralidoxime iodide and atropine, practically unlimited amounts of paraoxon and 217-AO could be given to rats without affecting the respiratory centre (Schaumann, 1959; 1960b).

When the lethal dose of an organophosphate was injected after pretreatment with pralidoxime iodide, death was due to the central inhibition of cholinesterase alone. Under these conditions, the enzyme activity found in the brains of mice was 1 to 5.5% of the control level, depending on the compound used (Schaumann, 1960b). This is the minimum amount of cholinesterase necessary to prevent the accumulation of toxic concentrations of acetylcholine in the central nervous system. However, if enough atropine is given to prevent the toxic effects of acetylcholine, the cholinesterase in the brains of rats and mice can apparently be inactivated completely without causing death.

It should perhaps be pointed out that this statement is not inconsistent with the assumption of cholinergic transmission within the central nervous system. Only the acetylcholine receptor, and not the cholinesterase, is primarily concerned in the process of transmission. The liberated acetylcholine which accumulates after the inhibition of the cholinesterase can be removed by diffusion (Ogston, 1955) and can be hydrolysed in the periphery which, due to the protective action of pralidoxime iodide, contains active enzyme.

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SCREENING OF CESTICIDAL COMPOUNDS ON A TAPEWORM *HYMENOLEPIS NANA IN VITRO*

BY

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A simple and convenient *in vitro* technique is described for the screening of compounds for action against *Hymenolepis nana* and probably many other intestinal worms. The results obtained from this test are in broad agreement with the findings of clinical experience and of a small series of *in vivo* tests. Among the substances tested, the most active ones were oil of chenopodium, dichlorophen, extract of cashew nut (*Anacardium occidentale*), antimony potassium tartrate, and BIQ 20 [eicosamethylenebis(isoquinolinium iodide)].

The testing of possible chemotherapeutic compounds upon helmintic infections in general is usually a cumbersome process which consumes great amounts of the chemical specimen, of animals and of time. In the present paper an *in vitro* technique is described for testing compounds on intestinal worms such as *Hymenolepis nana*; this technique is simple, and economical of time, animals and the specimen under test. It could be applied to many other intestinal worms. A preliminary notice of this work has been given elsewhere (Sen and Hawking, 1959).

METHODS

The worm selected for this work was *Hymenolepis nana*, which is a common tapeworm of mice, and which also occurs in man, especially in the Mediterranean area, Near East, and S. America; it can be transmitted directly by ova from mouse to mouse, since both stages of the worm (cysticercoid and tapeworm) occur in the same host. The worm was maintained in mice according to the technique described by Steward (1955). Ova were obtained from ripe segments of worms taken from infected mice. It was necessary to check under the microscope that the ova were mature since immature eggs will not infect mice. The eggs were freed from the segments of worms by light crushing under a cover slip, and they were then washed into boiled and cooled tap water. They were allowed to stand in water for 20 (or 44) hr. at room temperature (about 30°). The ova were then counted in a haemocytometer chamber, and an appropriate number (500 to 2,000) were given to mice by syringe and stomach tube.

Twenty to forty days after inoculation the mice were killed, having been starved for 24 hr. beforehand. The intestine was washed out, by inserting the needle of a syringe into the upper end and perfusing it with saline. If an isotonic solution buffered to be slightly acid (*p*H 6.8) was employed, the worms came out more readily. Alternatively, the small intestine freed from the mesentery was placed between two thick glass plates which were clamped together and examined by the naked eye or a binocular dissecting microscope. Worms *in situ* could be seen well by this technique, but they were difficult to recover from the intestine subsequently by perfusion. Newly weaned mice seem more susceptible than other mice. Some of the mice bred at this Institute contain natural infections of *H. nana* so that some older mice may possess an immunity from previous infections and some of the young mice may possess a partial immunity transmitted by immune mothers. Under good conditions the percentage of mice which later contained worms was about 80% and the number of worms was from 1 to 56 in each mouse. If only one worm was present 21 days after infection, it was usually a large one about 5 cm. long. If many worms were present they were small, from 0.5 to 1 cm. In this Institute at one season of the year, laboratory animals are often given cucumber as green food. There is some evidence that this had an anthelmintic action. Thus, of 7 control mice fed on stock diet, 6 (86%) were infected and they contained 3 to 13 worms each; while out of 5 mice fed with cucumber, only 2 were infected and they contained 3 and 5 small worms respectively. Accordingly, it is better to avoid a cucumber diet. A diet containing much potato seems to be favourable.

To expose the worms to compounds *in vitro*, the worms recovered from the intestine by perfusion were picked up free from mucus and other debris and transferred to two washes of Ringer solution. They were then placed in nutrient broth containing penicillin 500 units per ml. and streptomycin 0.1 mg. per ml.

The formula of the nutrient broth was as follows :

Peptone	10 g.
Yeast extract	2.5 g.
Sodium chloride	5 g.
Distilled water	1,000 ml.

The *p*H was adjusted to 8.5 by adding 2 ml. of 40% sodium hydroxide solution. After 0.5 to 1 hr. in nutrient broth at room temperature (30 to 34°) they were transferred to 3 ml. of the same medium in a suitable container. For this purpose small Kjeldahl's flasks of 10 ml. volume were used. Concentrations of penicillin as high as 2,000 units per ml. and of streptomycin as high as 1.0 mg./ml. were harmless to the worms. One or two worms were placed in each flask, which was then bunged. Suitable concentrations of the various compounds had been previously added to the nutrient broth in each flask. Since it was desired to make a broad survey of a large number of compounds, concentrations were chosen in multiples of 10. If the compound was insoluble in water, it was ground up, and a fine suspension was made by shaking in broth ; sometimes the compound was dissolved in alcohol before adding to broth. In these cases the maximum concentration of the compound depended on its solubility in the broth, and the concentrations recorded in the table are nominal ones, the actual concentration being lower. Preliminary experiments showed that concentrations of alcohol greater than 1% are harmful to the worms and such concentrations were avoided. The flasks were incubated at 37°. For examination they were placed in a horizontal position under a binocular dissecting microscope with low magnification where the movements of the worms could easily be observed. The worms of the control flasks with drug-free medium remained actively mobile for 5 days. The action of the drug, however, was read after 24 hr. because it is unlikely that a drug given by mouth would stay in the intestine even as long as 24 hr. Bacterial growth in the flask did not occur to any appreciable extent.

Some tests were made, for comparison, of the action of the drugs *in vivo*, using the technique of Steward (1955). Groups of mice were taken on the 14th day after infection. The drug was given in a single dose by oral catheter. The mice were starved on the second day and killed on the third day after treatment. The worms were collected from the intestine and scored by the number of worms present multiplied by a factor for the size of the worm (worms more than 3 cm., 20 ; about 2.5 cm., 10 ; about 1.2 cm., 5 ; about 0.6 cm., 0.5 ; less than 0.3 cm., 0.1). The results thus obtained were compared with those from a control group of untreated mice.

Results

The results obtained during the *in vitro* experiments are shown in Table I ; each figure is based on five separate experiments. The readings with carbon tetrachloride and tetrachlorethylene were very variable, ranging from 1:1,000 to

Table I

THE MINIMUM CONCENTRATIONS OF VARIOUS COMPOUNDS REQUIRED TO KILL *HYMENOLEPIS NANA* AT 37° IN 24 HR.

The extracts dichlorophen and oil of chenopodium were dissolved in alcohol and then diluted with water and Ringer solution. The solubility was greater than the minimum lethal concentration except for sulphadiazine (soluble 1 : 1,300), santonin (very slightly soluble), tetrachlorethylene, phenothiazine, pamaquin (insoluble), carbon tetrachloride (soluble 1 : 2,000).

Compound	Minimum Lethal Concentration
Male fern extract	1 : 100,000
Extract of cashew nut (ether extract of *Anacardium occidentale*)	1 : 1,000,000
Oil of chenopodium	1 : 10,000,000
Thymol	1 : 10,000
β-Naphthol	1 : 10,000
Arecoline	1 : 100,000
Santonin	1 : 1,000
Hexylresorcinol	1 : 100,000
Dichlorophen (Dicestal)	1 : 2,000,000
Carbon tetrachloride	1 : 10,000
Tetrachlorethylene	1 : 10,000
Phenothiazine	1 : 1,000
Mepacrine hydrochloride	1 : 100,000
Acriflavine	1 : 100,000
Chloroquine phosphate	1 : 100,000
Pentaquine phosphate	1 : 100,000
Pamaquin	1 : 100,000
Stilbamidine isethionate	1 : 1,000
Propamidine isethionate	1 : 1,000
Piperazine	1 : 1,000
Diethylcarbamazine	No action
Arsenamide [(*p*-carbamoylphenylarsinidenedithio)diacetic acid] ..	1 : 10,000
Melarsoprol (Mel B)	1 : 1,000
Antimony potassium tartrate (tartar emetic)	1 : 1,000,000
Copper sulphate	1 : 1,000
Sulphadiazine	No action
Iodochlorhydroxyquin (Vioform) ..	1 : 100,000
Bialamicol hydrochloride (camoform)	1 : 1,000
Lucanthone hydrochloride	1 : 10,000
Quinapyramine (Antrycide) ..	1 : 1,000
Prothidium (7-amino-2-(2-amino-6-methylpyrimidin-4-ylamino)-9-*p*-aminophenylphenanthridine 10,1′-dimethobromide	1 : 1,000
BIQ 20 [eicosamethylenebis(isoquinolinium iodide)]	1 : 1,000,000
Penicillin (1,000 units per ml.) ..	No action
Streptomycin (1 mg. per ml.) ..	,, ,,
Chloramphenicol ,, ...	,, ,,
Tetracycline ,, ...	,, ,,
Ethyl alcohol	1 : 50

1 : 100,000 in 11 experiments ; these results may be related to the insolubility of these compounds in watery solutions, although preliminary solution in alcohol or acetone did not make the results any more consistent.

The compounds tested can be divided into groups according to their ability to kill the worms *in vitro* in 24 hr. The most active compounds, which kill at a concentration of 1/million or less include oil of chenopodium (1 : 10 million), extract of cashew nut (*Anacardium occidentale*), dichlorophen, antimony potassium tartrate (tartar emetic) and BIQ 20 [eicosamethylenebis(isoquinolinium)]. The moderately active compounds (killing at 1/100,000 concentration) include male fern extract, arecoline, hexylresorcinol, mepacrine, acriflavine, chloroquine, pentaquine, pamaquine, and iodochlorhydroxyquin (Vioform). The other compounds tested are relatively or absolutely inactive against *Hymenolepis*.

A few experiments were carried out in mice to compare the *in vivo* action of several well-known anthelmintics with the findings obtained *in vitro*. The results are shown in Table II. The compounds which were active *in vivo* had already shown activity *in vitro* with the exception of tetrachlorethylene (which had given very irregular results *in vitro*).

TABLE II

THE ACTION OF CERTAIN DRUGS UPON *HYMENOLEPIS NANA IN VIVO*

Compound	Dose mg./kg.	No. of Mice Free of Worms / No. of Mice Treated	Average Score	Evaluation of Treatment
Male fern extract	750	4/5	1·1	Active
Arecoline	20	3/5	3·2	,,
Hexylresorcinol	500	4/5	1·7	,,
Dichlorophen	100	5/5	0	Very active
Tetrachlorethylene	1·5	3/5	4·2	Active
Mepacrine	300	4/5	0·4	Very active
Untreated controls	—	0/5	32·2	

DISCUSSION

The chemotherapy of helminthiasis has been surveyed in excellent reviews by Watkins (1958) and by Whitten (1956). Accordingly the present discussion will be limited to brief comments on certain aspects.

Technique of Testing.—Many techniques for testing anthelmintics have been described, but methods involving the survival or death of tapeworms *in vitro* have not been used. The method described above is simple and economical ; the worms from a few mice are sufficient to test many different drug-concentration combinations ; only a minute amount of chemical compound is required ; no previous toxicity tests are required. Theoretically, an *in vitro* method seems justified for screening compounds upon intestinal worms (although not upon parasites in the blood or tissues) since intestinal worms live in the lumen of the gut and anthelmintic drugs which have been given by mouth reach the parasites in the intestine without much opportunity for chemical modification. Of course, no single chemotherapeutic test can be guaranteed to detect 100% of active compounds, but as a compromise between expense and efficiency the present test seems good. The method is applicable to other parasites of the intestine and it is planned to apply it to some of the nematode worms. Preliminary experiments have shown that *Nippostrongylus muris* lives well for many days in simple media like Ringer-glucose plus antibiotics, and that the helminthicidal action of drugs upon it can readily be tested. When compounds have been selected by this test as having a high degree of activity compared with their probable toxicity, further investigation is of course needed by *in vivo* tests in mice infected with *Hymenolepis* and by detailed studies of oral toxicity.

Results of Testing.—The most active compounds, which kill in our test at a concentration of 1/million, include oil of chenopodium (1:10 million), extract of cashew nut, dichlorophen, antimony potassium tartrate and BIQ 20. Of these, oil of chenopodium is well known clinically for its action against ancylostomes and dichlorophen is used successfully for the treatment of tapeworms in dogs. Extracts of cashew nut have been tested clinically for anthelmintic action by Bhaduri, Chakravarti, Bandyopadhyay, Roy, and Arora (1958) with encouraging results ; they have also given further references. Antimony potassium tartrate is too toxic for this kind of therapy, and BIQ 20 is a new compound, synthesized by the workers of Allen & Hanburys and found to possess activity against the filarial worm *Litomosoides carinii* (Hawking and Terry, 1959); it will receive further investigation. The

moderately active compounds which kill at a concentration of 1/100,000 include:

male fern extract, hexylresorcinol and mepacrine	which are well-known remedies for human tapeworms.
arecoline	which has been used extensively for tapeworms in dogs, and which is an active depressant of the musculature of cestodes (Batham, 1946; Duguid and Heathcote, 1950a and b).
chloroquine	this is worth further investigation as a drug against tapeworms. It has been reported by Camero (1951) to be effective in removing *Taenia saginata* (7 cases described).
pentaquine and pamaquin	these are also worth further investigation, although their toxicity is greater than that of mepacrine and chloroquine, which would be a disadvantage.
iodochlorhydroxyquin	this would be worth investigating whether it acts on tapeworms as well as on amoebae.
acriflavine	this would presumably be too irritating and too toxic.

Tetrachlorethylene and carbon tetrachloride are difficult to test *in vitro* because of their insolubility and they gave irregular results, the minimum lethal concentrations varying from 1:1,000 to 1:100,000 in different experiments; but when tested *in vivo* tetrachlorethylene given in low dosage seemed moderately active. This is clearly one of the cases in which the *in vitro* test yields equivocal results and further investigation by *in vivo* tests is necessary.

The remaining compounds tested are inactive. In the case of phenothiazine, the inactivity may be due to the insolubility of the compound in our medium. Santonin, although clinically effective against *Ascaris*, is not effective in expelling tapeworms. The other compounds would not be expected to be active upon cestodes. On the whole, the results from our *in vitro* test are in agreement with those obtained by clinical experience.

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PHARMACOLOGICAL ACTIVITY OF AN AQUEOUS EXTRACT OF THE LEAVES OF THE MALAYAN RENGAS TREE *GLUTA RENGHAS*

BY

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A pharmacological analysis of an aqueous extract of the leaves of *Gluta renghas* has been carried out. It has been shown that the extract contained a heat-stable, anticholinesterase agent. A heart-stimulating substance was also shown to be present in the extract. Intravenous injection of the extract into the anaesthetized cat produced changes of arterial blood pressure, venous pressure and respiration which were reduced or abolished by vagotomy. The leaves contained approximately 0·1 μg./g. of 5-hydroxytryptamine-like activity.

"Rengas" is the name given by Malays to forest trees which contain a highly vesicant sap. The trees have been classified into five genera, all belonging to the family Anacardiaceae (Corner, 1940). The commonest Malayan variety is *Gluta renghas* and the vesicant property of the latex contained in its seeds has been attributed to the presence of a monoethenoid alkyl catechol, glutarenghol (Backer and Haack, 1941). In addition, the fruit of this tree has been claimed to cause severe gastro-intestinal irritation if eaten by man (Burkill, 1935). Aqueous extracts of the bark are reported to be lethal if swallowed and the exudate from the leaves and stems has been used as a poison in Malaya and Indonesia, but it is not known if any of these effects are also brought about by glutarenghol. The present experiments show that aqueous extracts of the leaves of *Gluta renghas* possess a number of pharmacological actions although, unlike the undiluted sap, they are not vesicant.

METHODS

Preparation of the Leaf Extract

Gluta renghas is a tall, evergreen forest tree with glabrous leaves and a white sap which darkens rapidly on exposure to air and light. The extract was prepared from freshly collected leaves at various stages of maturity (Fig. 1); these were decimated in a Kenmix and then weighed. The finely divided leaves were macerated with acid-washed sand and distilled water in a mortar. The resulting suspension was squeezed through several layers of cotton gauze to remove coarse particles and centrifuged at 3,000 r.p.m. for 30 min. The supernatant fluid was acid (*p*H 5.0 to 5.5) and before use the *p*H was adjusted to 7.4 with N/3 sodium hydroxide. The colour of the extract, which was stored at 4°, changed gradually from pale yellow to dark red-brown, and for this reason only extracts which were 48 hr. old, or less, were used in all experiments, unless otherwise stated.

a b

FIG. 1.—A single leaf (a) and a spray of leaves (b) of *Gluta renghas*.

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In some experiments a decoction was made by boiling the weighed, decimated leaves with distilled water for about 2 hr. The suspension was then filtered, centrifuged and neutralized in the manner described for the extract.

Dialysis of the extract or decoction was carried out, when required, by suspending about 50 ml. in a cellophane bag around which a stream of distilled water was passed for 48 hr. The dialysis bag had an average pore diameter of 48×10^{-7} mm. and it was freely permeable to trypan blue (mol. wt. 960).

Pharmacological Tests

Guinea-pig Ileum.—Freshly isolated segments of guinea-pig ileum were suspended in a 5 ml. bath filled with Tyrode solution at 37°. The solution was aerated with either a gas mixture of 95% oxygen and 5% carbon dioxide or air.

Rat Colon.—Segments of ascending rat colon were set up in a bath of 8 ml. Locke solution containing 1/8th of the usual amount of calcium chloride, at room temperature (28 to 30°). Atropine sulphate, sufficient to abolish the response of the colon to acetylcholine, was added to the bath before each addition of extract.

Rat Stomach.—Strips of muscle from the rat stomach were prepared according to the method described by Vane (1957). Each strip was suspended in an 8 ml. bath filled with Locke solution at 37°. The solution was aerated with air and hyoscine hydrobromide was added to the Locke solution to give a final concentration of 10^{-7}.

Isolated, Perfused Toad Heart.—Toad hearts were perfused through a Symes cannula with Ringer solution at room temperature.

Isolated, Perfused Mammalian Heart.—The hearts of guinea-pigs and rabbits were perfused with McEwen solution (1956), aerated with 95% oxygen and 5% carbon dioxide, at 37°. The solution was passed through a cannula inserted into the aorta and in some experiments the coronary outflow was measured.

Cat Blood Pressure and Respiration.—The effect of intravenous injections of the extract was investigated in cats anaesthetized with pentobarbitone sodium (45 mg./kg.) or chloralose (100 mg./kg.). In some experiments arterial blood pressure was measured by means of an arterial cannula inserted into the left common carotid artery and connected to a mercury manometer. In others, a heparinized polythene catheter filled with 2% sodium citrate solution was inserted into the right femoral artery and the blood pressure was recorded by means of a Statham strain-gauge pressure transducer, and a direct writing, Sanborn, 4-channel, physiological recorder. Central venous pressure was similarly recorded from a catheter threaded down the right external jugular vein, so that its tip lay at or near the junction of the superior vena cava with the right atrium. Lead II electrocardiograms were displayed on another channel of the recorder.

Respiratory movements were recorded either by a tambour and writing lever connected to a tracheal cannula or by a strain-gauge pressure transducer and catheter attached to a balloon inserted into the abdominal cavity.

Estimation of the Anticholinesterase Activity of the Extract.—This was done in two ways and the general procedure for both techniques has been described before (Lin, 1955). (*a*) The leaf extract was incubated at 38° for 1 hr. in a 0.134 M phosphate buffer solution (*p*H 7.2) together with a homogenate of guinea-pig brain and acetylcholine. The acetylcholine remaining at the end of the incubation was assayed on the toad rectus abdominis muscle suspended in eserinized Ringer solution at room temperature and aerated with air. For comparison, eserine salicylate was used in place of the extract. The brain homogenate was prepared by removing the cerebral hemispheres from a freshly killed guinea-pig and homogenizing 5 g. of tissue with 2 ml. phosphate buffer for about 10 min. in a Waring blender. The homogenate was made up to 30 ml. with phosphate buffer and thoroughly mixed. (*b*) The anticholinesterase activity of the extract was estimated manometrically in a Warburg apparatus at 38°. The reaction mixture in each vessel had a total volume of 5.0 ml. The cholinesterase was incubated with the extract for 45 to 60 min. before acetylcholine was added to the reaction mixture and hydrolysis of the acetylcholine was followed for a period of 120 min. The cholinesterase was provided either by a rat brain, homogenized and suspended in 24 ml. of bicarbonate Ringer solution, or by guinea-pig plasma diluted 1 in 10 with bicarbonate Ringer solution.

RESULTS

Effect of the Latex on the Skin

The vesicant properties of the sap were demonstrated on one of the authors by gently rubbing the cut end of a young stem on the forearm skin for approximately 10 sec. An irritant dermatitis resulted which persisted for three weeks and had to be treated clinically. In contrast, the leaf extract had no effect when applied topically to the forearm skin or to the shaved skin of the guinea-pig abdomen.

Pharmacological Actions of the Leaf Extract

Guinea-pig Ileum.—The extract caused a powerful contraction of the longitudinal muscle of the gut. The onset of contraction was delayed and occurred approximately 30 sec. after the extract was added to the bath. The contraction was usually well maintained even after the extract had been washed out, but it was inhibited completely by the addition of atropine to the bath (Fig. 2). After the extract had been washed out of the bath and the contraction had subsided, the response of the gut to acetylcholine was greater than it was

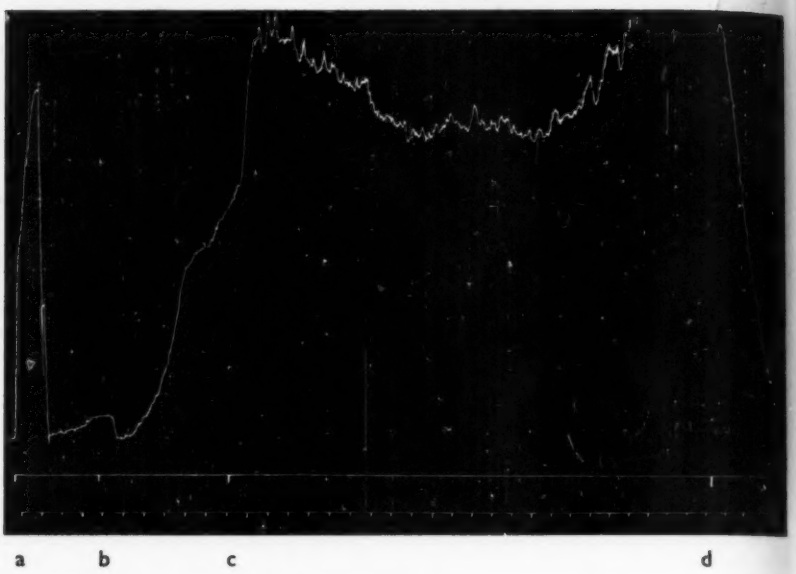

FIG. 2.—Effect of *Gluta renghas* leaf extract on the isolated guinea-pig ileum. At (a) 0·04 μg. acetylcholine and at (d) 2·0 μg. atropine sulphate were added to the bath. The effect of 0·5 ml. leaf extract (100 mg. fresh leaf tissue) is shown at (b), and at (c) the leaf extract was washed out of the bath. Time, 30 sec.

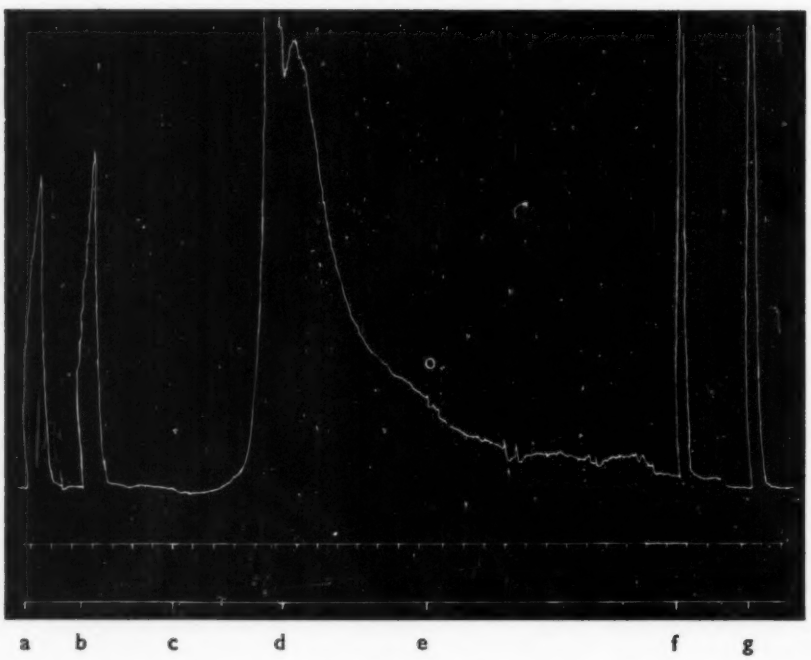

FIG. 3. — Response of the guinea-pig ileum to acetylcholine before and after addition of *Gluta renghas* leaf extract to the bath. Acetylcholine 0·2 μg. was added at (a) and (b), and 0·1 μg. at (f) and (g). At (c) 0·2 ml. extract (100 mg. leaf tissue) was added, and at (d) and (e) the bath was washed out with Tyrode solution. Between (f) and (g) there was an interval of 30 min. Time, 30 sec.

before the addition of the extract (Fig. 3). The agent, present in the extract, which caused contraction of the smooth muscle and which potentiated the effect of acetylcholine was stable to heat. A decoction of *Gluta renghas* leaves produced the same effect as the extract. The activity of the extract was not affected by boiling with N/3 sodium hydroxide for 1 min. and it was not lost during dialysis against distilled water for 48 hr.

Rat Colon.—Both an extract and a decoction of *Gluta renghas* leaves brought about a contraction of the atropinized rat colon and this response was reduced or abolished by the addition to the bath of (+)-2-bromolysergic acid diethylamide or methylergometrine tartrate (Methergin) in doses which abolished the response of the gut to 5-hydroxytryptamine (Fig. 4). The dialysed extract produced relaxation of the colon.

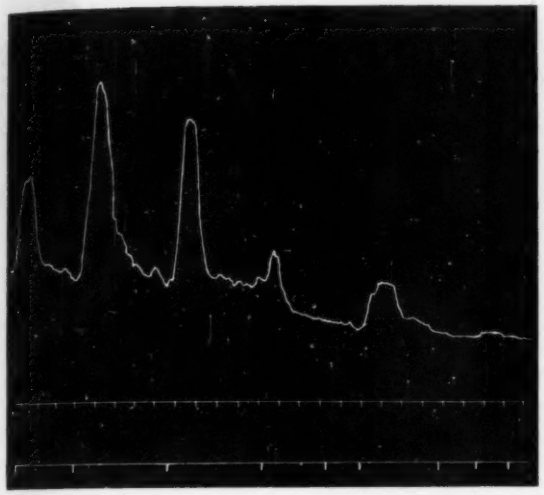

FIG. 4.—Contractions of the rat colon on the addition to the bath of the following: at (a) 0·2 ml. *Gluta renghas* leaf extract (10 mg. fresh leaves); at (b) 2·0 ng. 5-hydroxytryptamine; at (c) 0·3 ml. leaf extract (15 mg. leaf tissue); at (d) 10 μg. (+)-2-bromolysergic acid diethylamide which remained in the bath until it was washed out at (i); at (e) 2·0 ng. 5-hydroxytryptamine; at (f) 0·3 ml. leaf extract; at (g) 0·2 μg. atropine sulphate which remained in the bath until (i); at (h) 0·3 ml. leaf extract. Time, 1 min.

Rat Stomach.—The extract caused a contraction of the rat-stomach muscle, but this effect was not seen after the addition of 20 μg. methylergometrine to the bath. When assayed on the rat stomach preparation 1 g. leaf tissue was found to contain the mean equivalent activity of 110 ng. 5-hydroxytryptamine.

Toad Heart.—The experiment of Fig. 5 shows that 40 mg. leaf tissue had a marked stimulant action on the isolated toad heart. This effect could also be shown in the atropinized heart. The heart-stimulating substance was heat stable and resistant to the action of acid and alkali. It slowly passed through the wall of the dialysis bag into the surrounding water, but an extract which had been dialysed until it no longer caused contraction of the rat colon still stimulated the toad heart. Amounts of extract equivalent to as little as 4 mg. leaf tissue produced a large increase in the amplitude of the heart immediately after relatively large doses of 5-hydroxytryptamine (>5 μg.) had been shown to have no effect on the preparation. The action of the extract on the heart was not altered by the addition of methylergometrine to the perfusate either immediately before, or together with, the injection of the extract. Repeated, large doses of the extract (40 to 80 mg. leaf tissue) brought about a progressive increase in the tone of the heart.

Mammalian Heart.—Injection of the extract into the cannula of the isolated perfused guinea-pig or rabbit heart first increased the amplitude of contractions, then reduced it and finally stopped the heart in a state of systolic arrest (Fig. 6). During the period of cardiac stimulation the coronary outflow was increased.

Cat Blood Pressure and Respiration.—Intravenous injection of either the extract or the decoction, dialysed or undialysed, produced apnoea in the expiratory phase, a rise of venous pressure and an initial decrease in the heart rate (Fig. 7). The effect on arterial blood pressure was usually triphasic, consisting of a small rise, followed by a fall and then a rise above the pre-injection level. The secondary rise of arterial blood pressure was accompanied by an increased heart rate. After the injection of atropine, or when the extract was injected for the first time into the atropinized animal, the over-all response was similar but there was no slowing of the heart. When the vagi were sectioned, inhibition of respiration was no longer observed upon injecting the extract or decoction, and the rise of venous pressure was much reduced in magnitude. The response of the arterial blood pressure was either abolished or considerably

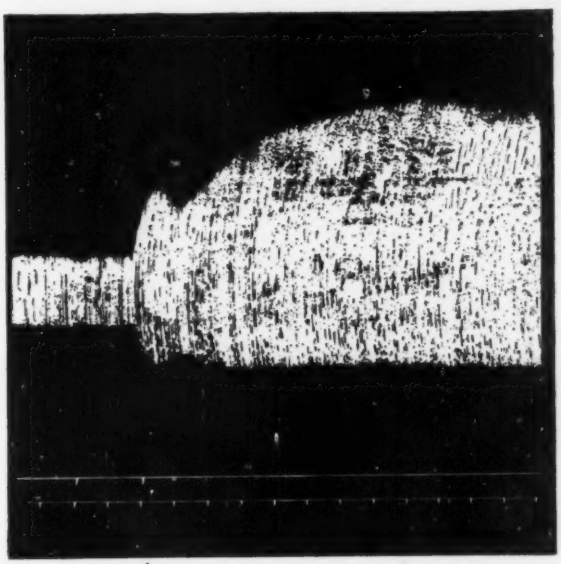

FIG. 5.—Stimulation of the isolated, perfused toad heart by 0·1 ml. *Gluta renghas* leaf extract (40 mg.) injected at (b). The effect of injecting 0·1 ml. Ringer solution into the Symes cannula is shown at (a). Time, 20 sec.

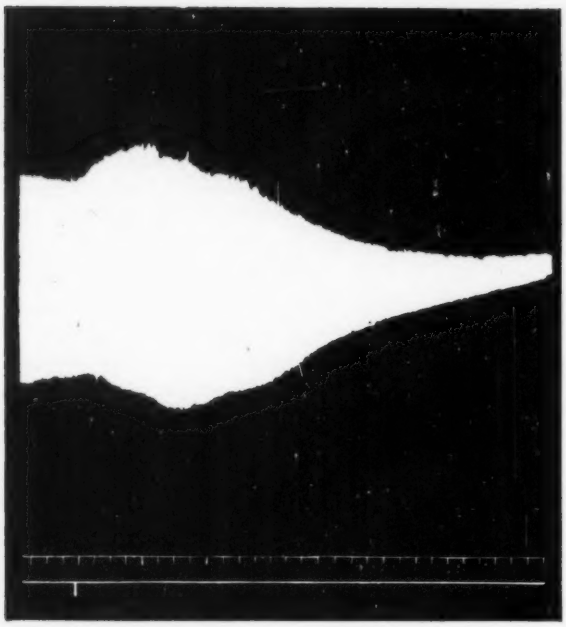

FIG. 6.—Stimulation of the isolated, perfused rabbit heart by 0·1 ml. *Gluta renghas* leaf extract (40 mg. leaf tissue) injected at the arrow. Time, 30 sec.

reduced, but an increased heart rate could still be observed. Injection of the extract into the previously vagotomized animal, which had not received any prior treatment with extract, produced a response similar to that shown in Fig. 7e. A dose slightly larger than that required to produce the apnoeic and depressor responses caused death from respiratory and circulatory failure in the anaesthetized animal with the vagi intact. Successive injections of the extract caused progressively greater disturbances of the rhythm of the heart beat and in the vagotomized animal the deterioration of the condition of the heart led to the death of the animal. The electrocardiogram showed that the disturbances of the heart rhythm consisted of periods of ventricular extrasystoles alternating with periods of complete cardiac arrest for about 6 sec. at a time.

Rabbit Eye.—No effect could be demonstrated on the diameter of the rabbit pupil when 1.0 ml. of the extract (750 mg. fresh leaves/ml.) was dropped into the eye, and the extract did not produce any lesion of the corneal or conjunctival surface.

Tachyphylaxis.—Tachyphylaxis was encountered with the guinea-pig ileum preparation, and on some occasions only a single response to the extract could be elicited from any one piece of gut. Re-

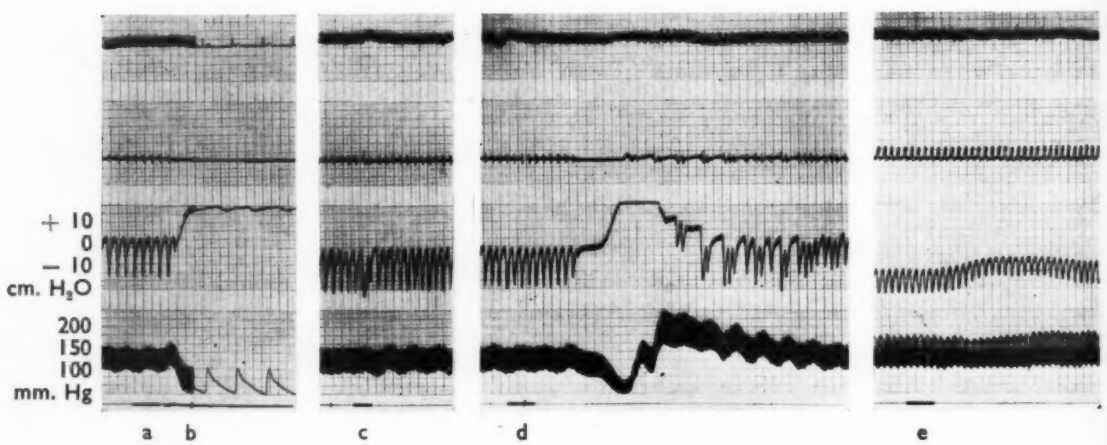

FIG. 7.—Cat, 3 kg., anaesthetized with chloralose. Records, from above downwards: electrocardiogram (lead II), intra-abdominal pressure (respiratory variations; inspiration ↑), central venous pressure, arterial blood pressure and signal/time-marker (1 sec.). Intravenous injection of 1·0 ml. *Gluta renghas* leaf extract (500 mg. fresh leaves) at (a), (d) and (e). At (b) the paper speed of the recorder was temporarily increased from 1 mm./sec. to 25 mm./sec. to show the electrocardiogram, and at (c) 1·0 μg. acetylcholine was injected intravenously. Between (b) and (c) 1·0 mg. atropine sulphate was injected intravenously and between (d) and (e) the vagi were cut in the neck.

peated injections of the extract at short intervals also produced tachyphylaxis of the blood-pressure and respiratory responses of the anaesthetized cat.

Pharmacological Activity of Stem Extracts

Extracts of young, green stems (30 mg. tissue) and also of old woody stems (50 mg. tissue) caused the isolated guinea-pig ileum to contract and this contraction was inhibited by the addition of atropine to the bath. The response of the gut to acetylcholine was enhanced by the extract. Stem extracts (170 mg./ml. stem tissue) also contracted the rat colon and the contraction was abolished by (+)-2-bromolysergic acid diethylamide.

Anticholinesterase Activity of the Leaf Extract

Effect of Incubation of Acetylcholine with Guinea-pig Brain Cholinesterase in the Presence of the Extract.—The experiments carried out on the isolated guinea-pig ileum suggested that the leaf extract might possess anticholinesterase activity. Table I presents the results of one of five experiments which were designed to test this. The Table shows that 1.0 g. leaf tissue was approximately half as effective in preventing hydrolysis of acetylcholine as was 250 μg. eserine salicylate; 250 mg. leaf tissue conferred no measurable protection against hydrolysis, and in other experiments 500 mg. leaf tissue was also without effect.

Manometric Estimation of the Anticholinesterase Activity of the Leaf Extract.—Five experiments were carried out using the Warburg apparatus, and in all instances inhibition of brain-cholinesterase activity was produced by the extract. An example of the results obtained is given in Table II, which shows that 818 mg. leaf tissue had almost the same inhibitory effect as 250 μg. eserine salicylate and that 409 mg. caused only slightly less inhibition (93% as opposed to 99%) than did 818 mg. leaf tissue. Similar results were obtained when a decoction of leaves was used in place of the extract.

TABLE I

INHIBITORY EFFECT OF *GLUTA RENGHAS* LEAF EXTRACT ON THE HYDROLYSIS OF ACETYLCHOLINE BY GUINEA-PIG BRAIN CHOLINESTERASE

Flask Contents	Flask No.							
	1	2	3	4	5	6	7	8
Guinea-pig brain homogenate, 1·0 ml.	+	+	+	+	+	+	+	+
Phosphate buffer, 2·0 ml.	+	+	+	+	+	+	+	+
Acetylcholine (50 μg.), 1·0 ml. of 0·005% solution	+	+	+	+	+	+	+	+
Eserine salicylate (250 μg.), 0·05 ml. of 0·5% solution	0	0	+	+	0	0	0	0
Gluta renghas leaf extract (250 mg./ml.), ml.	0	0	0	0	4	4	1	1
Distilled water, ml.	4·0	4·0	3·95	3·95	0	0	3·0	3·0
Acetylcholine recovered; estimated by assay on frog rectus abdominis muscle, μg.	<1·0	<1·0	45·0	45·0	25·0	23·0	<1·0	<1·0

TABLE II

RATE OF HYDROLYSIS OF ACETYLCHOLINE BY RAT-BRAIN CHOLINESTERASE IN THE PRESENCE OF *GLUTA RENGHAS* LEAF EXTRACT

Flask Contents	Manometer No.					
	1	2	3	4	5	6
Rat brain homogenate, 0·5 ml.	+	+	+	+	+	+
Bicarbonate buffer, 2·0 ml.	+	+	+	+	+	+
Acetylcholine (5·0 mg.), 0·5 ml. of 1·0% solution	+	+	+	+	+	+
Eserine salicylate (250 μg.), 0·05 ml. of 0·5% solution	0	+	0	0	0	0
Gluta renghas leaf extract (409 mg./ml.), ml.	0	0	1·0	1·0	2·0	2·0
Distilled water, ml.	2·0	1·95	1·0	1·0	0	0
Rate of release of carbon dioxide, μl./hr.	318·1	7·3	28·0	28·5	11·8	12·1
Inhibition of enzymic hydrolysis, %	0	100	93·3	93·2	98·6	98·5

TABLE III

RATE OF HYDROLYSIS OF ACETYLCHOLINE BY GUINEA-PIG PLASMA CHOLINESTERASE IN THE PRESENCE OF (a) AN EXTRACT AND (b) A DECOCTION OF *GLUTA RENGHAS* LEAVES

Flask Contents	Manometer No.					
	1	2	3	4	5	6
Guinea-pig plasma, 0·5 ml.	+	+	+	+	+	+
Bicarbonate buffer, 2·0 ml.	+	+	+	+	+	+
Acetylcholine (30 mg.), 0·5 ml. of 6·0% solution	+	+	+	+	+	+
Eserine salicylate (250 μg.), 0·05 ml. of 0·5% solution ..	0	+	0	0	0	0
(a) Extract (400 mg./ml.), 2·0 ml.	0	0	0	0	+	+
(b) Decoction (350 mg./ml.), 2·0 ml.	0	0	+	+	0	0
Distilled water, ml.	2·0	1·95	0	0	0	0
Rate of release of carbon dioxide, μl./hr.	128·7	22·2	40·3	43·5	23·7	24·3
Inhibition of enzymic hydrolysis, %	0	100	82·5	80·0	98·6	98·0

In three separate experiments guinea-pig plasma was used as the source of cholinesterase and the results of one of these experiments are shown in Table III. In all three experiments the rate of hydrolysis of acetylcholine by plasma was reduced by both the extract and the decoction of leaves. In the example given (Table III) the decoction was prepared from dried leaves which had been kept in the laboratory for one month after their collection from the tree.

The effect of 0.5 ml. of a 2.0% solution of catechol on the rate of hydrolysis of acetylcholine in the presence of rat-brain cholinesterase was tested in two experiments. In one of them catechol reduced the rate of enzymic hydrolysis of acetylcholine by 14%, and in the other experiment by 30%.

DISCUSSION

The evidence collected shows that the leaves of *Gluta renghas* contain an anticholinesterase substance. Aqueous extracts of the leaves reduced the rate of hydrolysis of acetylcholine by both " true " and " pseudo " cholinesterase *in vitro*, and experiments with the isolated guinea-pig ileum suggested that the extract potentiated the action of acetylcholine present in the gut wall, rather than caused a direct contraction of the smooth muscle. The present experiments have not provided any information about the nature of the anticholinesterase agent, other than that it is heat stable, resistant to the action of alkali and not readily dialysable. 409 mg. fresh leaf tissue reduced the rate of enzymic hydrolysis of acetylcholine by 93%, whereas 10 mg. pure catechol produced, at the most, only 30% inhibition of cholinesterase activity. The fresh leaves consisted largely of water (57% by weight) and fibrous tissue, and it is unlikely that as much as 10 mg. of pure catechol could be contained in 409 mg. fresh leaves. However, it is possible that the leaves contain glutarenghol or a related substance and that the alkylated catechols have a greater anticholinesterase activity than catechol itself.

The leaf extract also contained a powerful toad-heart stimulating substance which was resistant to the action of heat, acid, and alkali. The effect of the extract on the heart was not altered by atropinization, which suggests that acetylcholine was not involved in the response. The presence of a similar substance in the Malayan Jack-fruit seed has been reported by Lin (1955), but, in the present experiments, the leaf extract also stimulated the isolated mammalian heart. The increased tone of the toad heart, brought about by the addition of large doses of leaf extract to the perfusate, and the arrest of the mammalian heart after an initial phase of stimulation, by relatively small doses of extract, are actions which resemble those of some cardiac glycosides.

In the anaesthetized, intact animal, the leaf extract produced a fall of arterial blood pressure and also inhibition of respiration when injected intravenously. The afferent pathway for the depressor and apnoeic responses appeared to be the vagi, and in this respect the effects of the leaf extract resemble those produced by veratridine, 5-hydroxytryptamine, the amidines, and a number of other substances (Dawes and Comroe, 1954). The bradycardia which accompanied the fall of arterial blood pressure must have been associated with the release of acetylcholine at vagal nerve endings in the heart because it was abolished by atropine. It seems likely that this latter action

of the extract can be identified with the anti-cholinesterase activity described above, but the alternative possibility, that the bradycardia was brought about reflexly through an increased vagal tone, cannot be excluded by the experiments reported here. Since the depressor response could be demonstrated in the atropinized animal, in which the extract did not cause bradycardia, it could not have been entirely a consequence of the slowing of the heart. The increased heart rate which accompanied the secondary rise of arterial blood pressure and which could be shown to occur after vagotomy can be compared with the stimulation of the isolated mammalian heart by the extract. The disturbances in the electrocardiogram and the general deterioration of the condition of the heart, which caused the death of the vagotomized animal, are also reminiscent of the effect of the extract on the isolated mammalian heart. An interesting feature of the present results has been the rise of central venous pressure which occurred simultaneously with the cardiac slowing, fall of arterial blood pressure, and inhibition of respiration. It seems unlikely that this was a secondary effect due to either slowing of the heart, fall of blood pressure or inhibition of respiration, because Fig. 7 shows that the venous pressure remained high after respiration had started again and when the arterial blood pressure and heart rate had returned to or above their pre-injection levels. It is unlikely also that tachyphylaxis could account for the effects of vagotomy and atropine on the blood pressure, heart rate, and respiratory responses of the anaesthetized animal to the extract, because initial injection of the extract into the atropinized, vagotomized animal did not produce the responses shown in Figs. 7a and 7d.

Experiments with the rat colon and the rat stomach showed that the leaf extract possessed 5-hydroxytryptamine-like activity. It is possible that the 5-hydroxytryptamine-like activity of the extract contributed to its stimulant action on the isolated amphibian heart, although a direct comparison of the effects of the extract with those of 5-hydroxytryptamine, on the same heart preparation, suggested that this was not the case. The fact that methylergometrine did not alter the action of leaf extract on the toad heart, and that dialysed extracts could still stimulate the heart, also speaks against the identification of the heart-stimulating substance with 5-hydroxytryptamine. The participation of 5-hydroxytryptamine in the production of the apnoeic and depressor responses can be excluded since dialysed extracts, which had no effect on the rat colon or rat stomach preparation, were able to elicit these responses when injected intravenously into the anaesthetized cat.

The experiments described here have confirmed that *Gluta renghas* produces a vesicant latex (Backer and Haack, 1941), and it is not unreasonable to suppose that the action of the extract on isolated segments of mammalian gut could account, in part, for earlier claims that the fruit of this tree causes severe gastro-intestinal irritation (Burkill, 1935). The finding that relatively small doses of leaf extract caused the death of the anaesthetized cat from circulatory and respiratory failure is in accord with reports that extracts of the leaves and stems are lethal to man (Burkill, 1935).

We wish to record our gratitude to Professor D. W. Gould, of the Department of Physiology, University of Malaya, for making the electro-manometric measurements of blood pressure and respiration for us. We are also very grateful to Professor H. B. Gilliland, of the Department of Botany, for identifying the tree and for assistance in the collection of material for analysis. Our thanks are also due to Mr. J. Chua, who gave us technical assistance in all experiments.

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THE RESPONSE OF ISOLATED RABBIT ATRIA TO ACONITINE

BY

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The arrhythmias observed in isolated rabbit atria appear to be due to two actions of aconitine. One is an apparent direct stimulant effect on the sino-atrial node and the other is a depressant effect on the processes involved in intra-atrial propagation of impulses. Acetylcholine has either an inhibitory or stimulatory effect on atria pretreated with aconitine because it antagonizes the actions of aconitine both on the sinus node and on conduction. When conduction block is not apparent, acetylcholine depresses the atrial rate. On the other hand, when conduction is markedly depressed, acetylcholine increases the atrial rate.

Atrial flutter and fibrillation can be produced *in vivo* by the topical application of aconitine to the atria of the dog. It is believed that these arrhythmias are due to the rapid discharge of an ectopic focus of activity (Scherf, Romano, and Terranova, 1948; Prinzmetal, Corday, Brill, Oblath, and Kruger, 1952).

Acetylcholine is known to favour the establishment of atrial fibrillation after the local application of aconitine to atrial tissue. For instance, Scherf *et al.* (1948) showed that vagal stimulation converts aconitine-induced flutter to fibrillation, and Burn, Vaughan Williams, and Walker (1956) found that it was necessary to add acetylcholine to the blood to produce fibrillation with aconitine in the dog heart-lung preparation.

This study was undertaken to define the nature of atrial arrhythmias induced by aconitine in isolated mammalian atria and to determine the effects of acetylcholine on the response. A preliminary report of these results was made at the meeting of the American Society for Pharmacology and Experimental Therapeutics, Philadelphia, Pennsylvania, April, 1958.

METHODS

Male rabbits of the New Zealand strain were used. Immediately after stunning an animal by a blow on the back of the head, the thorax was opened and the entire heart removed and placed in oxygenated nutrient fluid at room temperature. The atria were carefully dissected from the ventricles by cutting along the atrio-ventricular junction and through the inter-auricular septum, and then were cleared of adjacent tissue and suspended vertically in a muscle chamber which was located in a larger thermostatically controlled water bath. The tip of the left atrium was anchored to the tissue holder, and the tip of the right atrium was attached to a silk thread leading to a force displacement transducer. The diastolic tension was arbitrarily set at 1 g. Action potentials were obtained with two unipolar silver electrodes. One electrode was placed on the right atrial appendage, another on the left atrial appendage, and an indifferent electrode was placed in the bath remote from the atria. The action potentials and isometric contractions were recorded on a four-channel Sanborn Polyviso Recorder. The working volume of the muscle chamber was 100 ml.

The composition of the tissue medium in mmol./l. was as follows: NaCl, 119.7; KCl, 5.6; $MgCl_2$, 2.1; $CaCl_2$, 2.1; $NaHCO_3$, 17.0; and glucose 9.0. The *p*H of this fluid aerated with 95% oxygen and 5% carbon dioxide at 37.5° was 7.4.

Solutions of aconitine were prepared by dissolving 5 mg. of pure aconitine crystals (S. B. Penick and Co., New York, N.Y.) in 50 ml. of distilled water containing one drop of N hydrochloric acid. Since aconitine solutions are unstable, fresh solutions were made on the day of each experiment.

RESULTS

General Response to Aconitine

Control experiments carried out for periods of from 5 to 6 hr. showed that the spontaneous rate of the isolated atria remained the same or showed a slight progressive decrease, and that there was a one-to-one relationship between the action potentials and the isometric contractions throughout the test period (Fig. 1). When a basis for comparison had been established, aconitine was added in various concentrations. In a concentration of 0.01 γ/ml. aconitine had no effect on the atria in 6 tests. With

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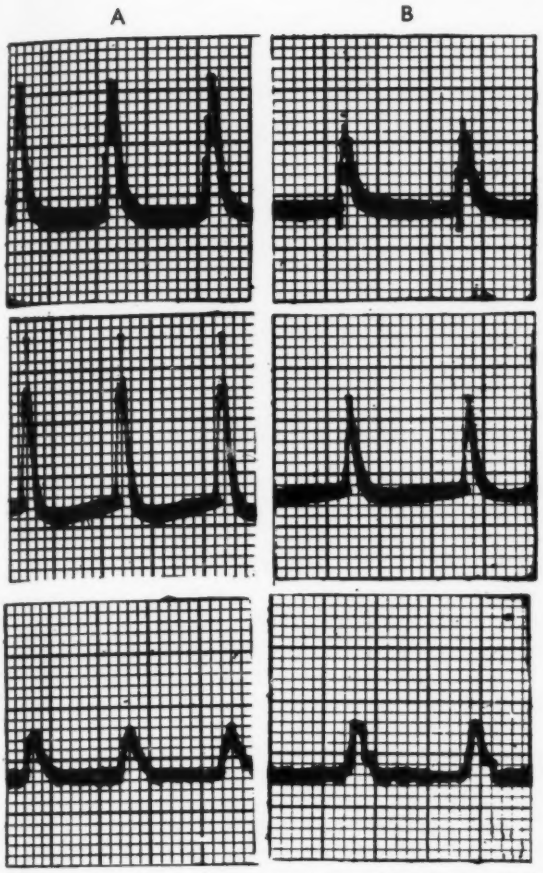

FIG. 1.—*Recordings from an Untreated Spontaneously Beating Isolated Rabbit Atria.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Record obtained at the beginning of the observation period; (B) record obtained at the end of 5 hr.; (B) shows a decrease in the spontaneous rate and in the size of the action potentials.

higher concentrations of aconitine, a triphasic response was produced. The first phase usually consisted of a progressive acceleration of the spontaneous rate. Intermittent bouts of tachycardia were occasionally observed before the maximal rate was reached. The second phase consisted of (*a*) an overall slowing in the rate, (*b*) intermittent periods of rapid and slow activity, (*c*) a loss of a one-to-one relationship between the action potentials recorded from each atrium, and between the action potentials and the contractions, (*d*) variations in the amplitude of the contractions, and (*e*) variations in the amplitude and shape of the action potentials. The onset of the third phase was manifested by apparent electrical and mechanical arrest of the atria. Four atria were observed for 40 min. after activity stopped. Two atria remained quiescent throughout this period, while occasional transient bursts of electrical activity were recorded from the right atrial appendage in the other two.

The effects of aconitine on the atria were reversed by numerous replacements of the nutrient fluid.

Aconitine (0.04 μg./ml.) produced only changes described as the first phase. When the concentration was increased to 0.16 μg./ml. the first and second phases were always seen and in 2 of 4 experiments the third phase was also observed. A concentration of 1 μg./ml. always produced a triphasic response (Fig. 2). The results are summarized in Table I.

Site of Action of Aconitine

A second series of experiments was carried out to determine whether aconitine acts during the initial phase by establishing an ectopic pacemaker, or by an action involving the sino-atrial node. To distinguish between these possibilities, experiments were conducted on isolated left atria, which did not beat spontaneously but responded to electrical stimuli. When stimulation was discontinued, the

TABLE I

EFFECTS OF GRADED DOSES OF ACONITINE ON ISOLATED RABBIT ATRIA

To estimate onset the aconitine response was considered to have begun when the atrial rate was increased by at least 20 beats/min.

No. of Atria	Aconitine μg./ml.	Control Rate Beats/Min.		Incidence of Response	Onset of Response Min. Range	Max. Rate Beats/Min.		Incidence of Arrest	Time for Arrest—Min.	
		Mean	Range			Mean	Range		Mean	Range
6	0·01	140	128–160	0/6				0/6		
4	0·02	130	120–140	2/4	5–10	340	320–360	0/4		
5	0·04	126	120–140	5/5	4–35	288	210–380	0/5		
4	0·16	145	120–160	4/4	2–10	365	340–420	2/4	50	40–60
5	1·0	135	120–160	5/5	1–5	360	300–480	5/5	36	20–52

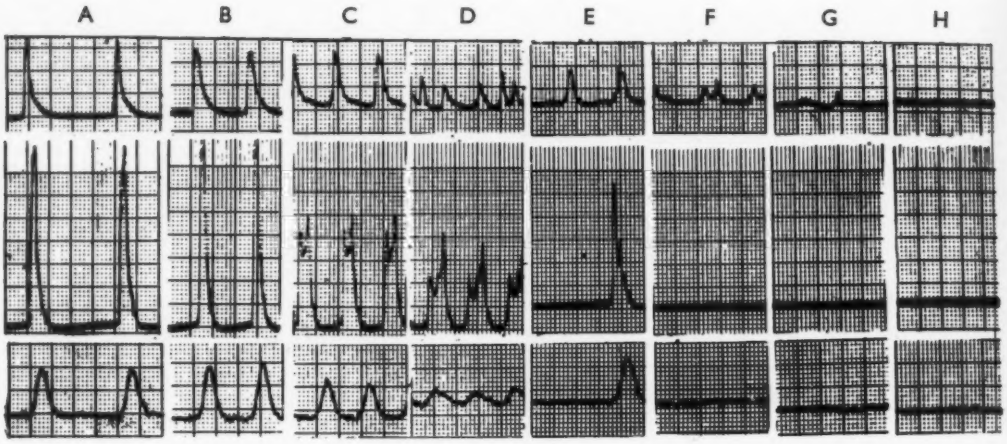

FIG. 2.—*Response of Isolated Rabbit Atria to Aconitine.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—50 mm./sec.). (A) Control; (B, C) records taken 2 and 4 min. after the addition of aconitine (1.0 μg./ml.) show the tachycardia characteristic of the first phase of the aconitine response; (D, E, F, G) records taken 6, 8, 10, and 15 min. after aconitine show interference with the propagation of action potential which characterizes the second phase of the aconitine response; (H) shows electrical and mechanical arrest of the atria characteristic of the third phase.

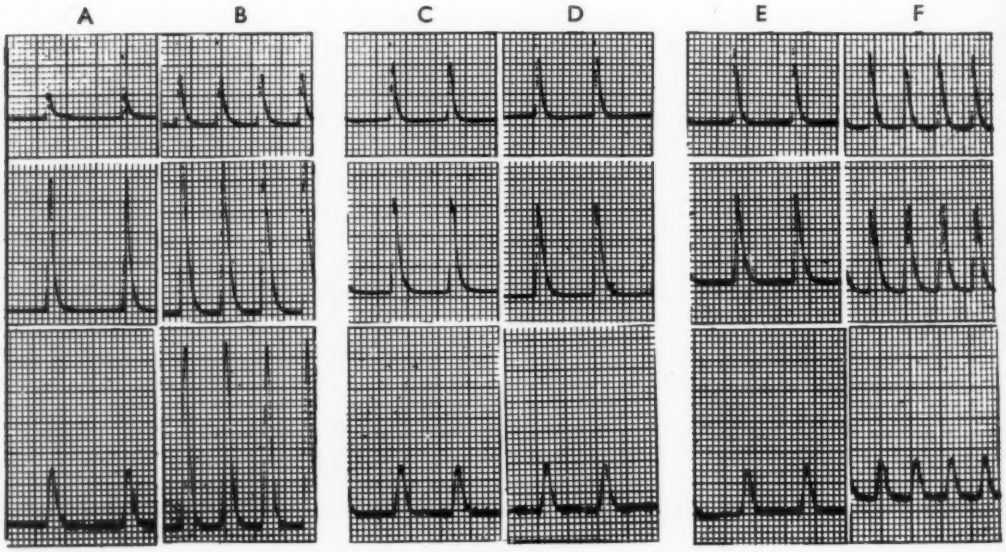

FIG. 3.—*The Effect of Hexamethonium on Aconitine-induced Atrial Tachycardia.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Control; (B) tachycardia induced by nicotine (9 μg./ml.); (C) record taken 10 min. after the removal of nicotine and the addition of hexamethonium chloride (0.06 mg./ml.) ; at this time, nicotine (9 μg./ml.) was again added to the bath; (D) block of the nicotine response by hexamethonium; (E) record taken after 9 washes and the subsequent addition of hexamethonium chloride (0.06 mg./ml.) ; at this time aconitine (0.04 mg./ml.) was added to the bath; (F) record taken 15 min. later shows hexamethonium does not block the tachycardia induced by aconitine.

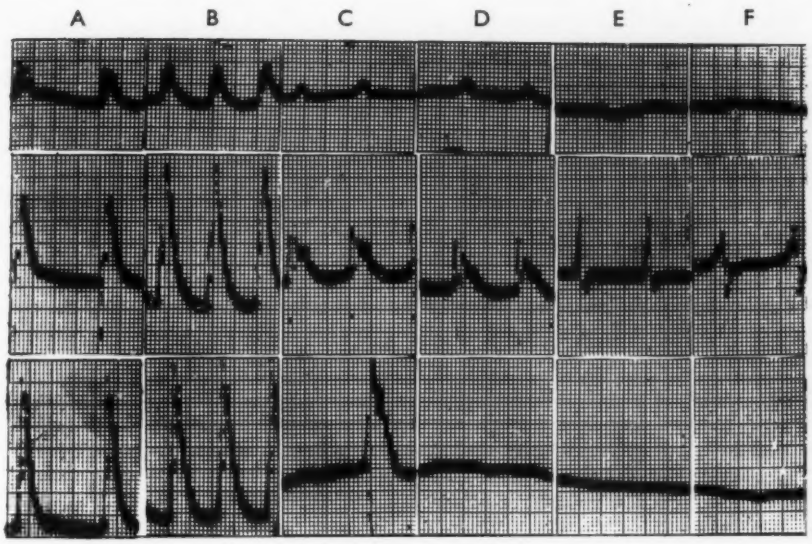

FIG. 4.—*Persistence of Electrical Activity in Atria Exposed to Aconitine.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from tissue in relatively close proximity to the sinus node; bottom tracing, electrograms from the left atrial appendage. (Paper speed—25 mm./sec.). (A) Control; (B) shows aconitine-induced tachycardia; (C, D, E, F) show the persistence of electrical activity in tissue near the sinus node and the progressive failure of the action potentials to be propagated to the appendages.

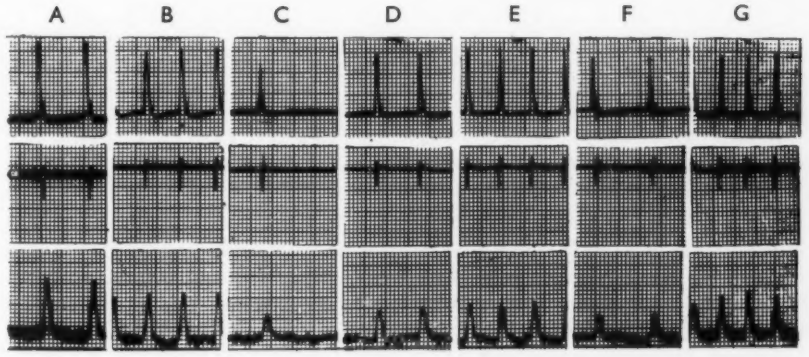

FIG. 5.—*Effects of Acetylcholine in Aconitine-induced Tachycardia.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Control; (B) tachycardia induced by aconitine; (C) shows the slowing of the rate immediately after the addition of acetylcholine (0.5 μg./ml.); (D) shows partial recovery of the rate 15 min. after acetylcholine was added; (E) shows complete recovery of the rate 20 min. after acetylcholine; (F) shows slowing of the rate after a subsequent addition of acetylcholine (0.5 μg./ml.); (G) shows recovery of the rate 5 min. after acetylcholine was added.

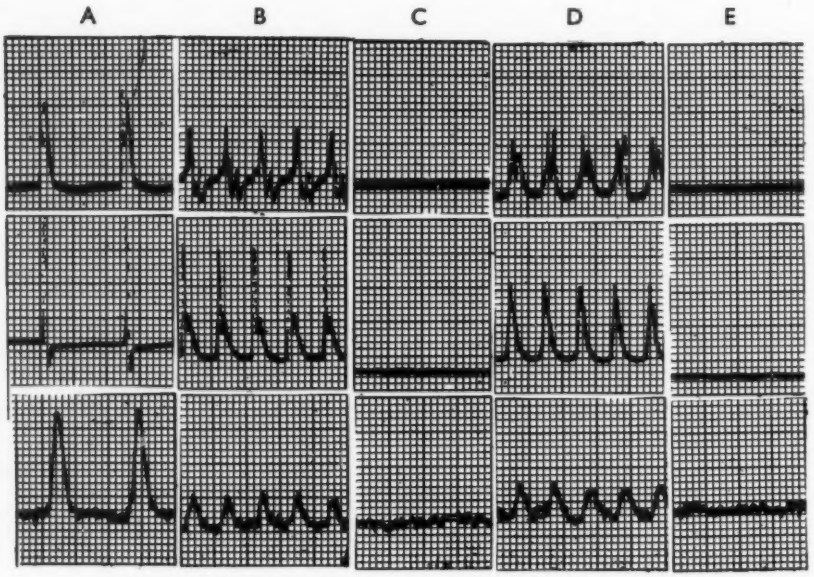

FIG. 6.—*The Effects of Acetylcholine in Atria Arrested by Aconitine.* Top tracing, electrograms from the right atrial appendage; middle tracing, electrograms from the left atrial appendage; bottom tracing, mechanograms. (Paper speed—25 mm./sec.). (A) Control; (B) shows aconitine-induced tachycardia; (C) shows arrest of the atria; (D) shows the immediate restarting of the atria by acetylcholine (0.05 μg./ml.); (E) shows arrest of the atria after a single replacement of the bath fluid.

atria stopped contracting. Aconitine (0.04 μg./ml.) produced no detectable electrical or mechanical activity in these atria during a 1 hr. observation period. At the end of the hr. the nutrient fluid was replaced, and after 5 min. the concentration of aconitine in the bath was increased to 1.0 γ/ml. Even so, no activity was observed in the next 25 to 30 min. The results suggest that aconitine was not establishing an ectopic pacemaker and so was affecting the sino-atrial node directly or indirectly.

It was possible that aconitine accelerated the atria by stimulation of sympathetic ganglia or atrial chromaffin tissue. The presence of sympathetic ganglia or chromaffin tissue in rabbit atria has been demonstrated by Kottegoda (1953), who reported that nicotine produced an increase in the rate of isolated atria which could be abolished by ganglionic blocking agents. It was found in 5 experiments that hexamethonium chloride (0.06 mg./ml.) or tetraethylammonium chloride (0.3 mg./ml.), which were shown in each test to block the tachycardia induced by nicotine (9 μg./ml.), did not modify the atrial tachycardia induced by aconitine. The results of one of these experiments are shown in Fig. 3.

It can be seen in Fig. 2 that conduction is impaired during the second phase of the aconitine response. The inability of the atria to conduct impulses well was found to be the cause of the relative inactivity of the atria during the third phase. This was determined in 4 experiments in which action potentials were recorded from each atrial appendage and from tissue in close proximity to the sino-atrial node. Fig. 4 shows that action potentials were recorded from the region of the node when no activity was recorded from the appendages.

The Effects of Acetylcholine on the Aconitine Response

During the first phase of the aconitine response, when the spontaneous rate was greater, acetylcholine (0.5 to 2.0 μg./ml.) depressed, for varying periods of time, the fast auricular rates induced by aconitine. A subsequent addition of acetylcholine was not as effective in depressing the rate and the effect did not last as long (Fig. 5). This antagonism by acetylcholine was seen when the concentration of aconitine in the bath was 0.04 or 1.0 μg./ml. Acetylcholine (0.05 to 2.0 μg./ml.) also antagonizes the depressant effects of aconitine on conduction. This can be seen in Fig. 6. In most instances, restarting of the atria was characterized by chaotic electrical activity and irregular contractions.

Physostigmine sulphate (0.05 μg./ml.) added during the third phase of the aconitine response

produced after a latent period of 4 to 15 min. the same effect as added acetylcholine. The latent period was presumably the time required to build up an effective concentration of acetylcholine. Atropine sulphate (0.1 to 0.2 μg./ml.) abolished the effects of acetylcholine or physostigmine. The results indicate that atropine can block the excitatory effects of endogenous acetylcholine in atria pretreated with aconitine. Webb (1950) showed that the depressant effect of endogenous acetylcholine could also be blocked by atropine; it was therefore assumed that pretreating atria with atropine would reveal whether or not the acetylcholine normally present in the atria plays a role in the genesis or maintenance of the aconitine response. In tests on 6 auricles, aconitine (0.04 to 1.0 μg./ml.) was added to the bath 10 min. after the addition of atropine (0.1 to 0.2 μg./ml.). The aconitine response was not modified. These experiments strongly suggest that the acetylcholine normally present in isolated atrial tissue is not at all concerned with the response of rabbit atria to aconitine.

Discussion

The experimental evidence obtained in this study indicates that aconitine-induced arrhythmias in isolated rabbit atria are due to stimulation of the sinus node and to interference with the propagation of impulses. The tachycardia observed after aconitine is attributed to an effect on the pacemaker, since isolated left atria which are presumably devoid of sinus nodal tissue do not respond to aconitine in concentrations that stimulate the paired atria. Furthermore, this effect on the pacemaker appears to be a direct action and not due to a nicotine-like stimulation of sympathetic ganglia of chromaffin tissue, since the response to aconitine is unaltered by ganglionic blocking drugs.

The depressant effects of aconitine on conduction are shown by the loss of a one-to-one relationship between the action potentials recorded from each atrial appendage and by the persistence of electrical activity in tissue in close proximity to the sinus node at a time when action potentials cannot be recorded from either appendage. This effect on conduction occurs with higher doses of aconitine than are required to cause atrial tachycardia and can be reversed by washing out the aconitine or by acetylcholine. The ability of acetylcholine to facilitate intra-atrial conduction when intra-atrial block is present has long been recognized (Lewis and Drury, 1923; Drury, 1925–26).

When atria depressed by aconitine were reactivated by acetylcholine, the electrical activity and contractions were irregular. It is evident that aconitine has a disproportionate depressant effect on the ability of atrial cells to conduct impulses, as shown by notching of the action potentials and the appearance of action potentials from one part of the atria when they are not being recorded from another part. Therefore, the arrested myocardium can be considered to be comprised of cells which have been depressed to varying degrees by aconitine. It would be expected that myocardial cells so depressed would vary at any one instance in their ability to propagate an impulse after the addition of acetylcholine. Under these conditions, re-entry of impulses is favoured. Furthermore, because of the marked acceleration of the repolarization process due to acetylcholine (Brooks, Hoffman, Suckling, and Orias, 1955; Hoffman and Sucking, 1953), the myocardial units would be capable of responding to greater rates of stimulation, therefore electrograms consisting of irregular action potentials could be expected.

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ANAESTHESIA IN NEW-BORN ANIMALS

BY

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Pentobarbitone was more toxic to new-born than to adult rabbits and rats, produced a longer loss of righting reflex in new-born animals but did not anaesthetize them effectively in less than toxic doses. Urethane did not anaesthetize new-born animals in doses which anaesthetized adults. Ether produced loss of righting reflex at lower concentrations for new-born than for adults, but the new-born animals became anaesthetized more slowly.

The responses of new-born animals to various drugs are known to differ from those of adults of the same species (Reiss and Haurowitz, 1929 ; Eddy, 1939 ; Chesler, Labelle and Himwich, 1942 ; Chen and Robbins, 1944) and it is not surprising, therefore, to find that they differ from adults in their responses to anaesthetic drugs. An appreciation of these differences is of importance to those working with new-born animals.

Homburger, Etsten and Himwich (1947) found that doses of pentobarbitone (40 to 50 mg./kg.), which killed rats less than 24 hr. old, produced a loss of righting reflex for only a few hours in adult rats. The sleeping time of new-born mice (Jondorf, Maickel and Brodie, 1959) which had received hexobarbitone was much longer than that of adult mice which had received the same dose. Fazekas, Alexander and Himwich (1941) found that new-born rats took longer to die than adults when breathing nitrous oxide or cyclopropane, although the time of arrest of respiration when breathing air containing an unstated concentration of chloroform vapour did not differ between newborn mice and mice older than 18 days (Reiss and Haurowitz, 1929).

While much of the evidence quoted above refers to the lethal effects of anaesthetic drugs, this paper is concerned with a comparison of some non-lethal effects of urethane, pentobarbitone and ether in new-born and adult rats and rabbits.

METHODS

Animals were exposed to ether in a 30 l. observation chamber at a controlled environmental temperature. Air was blown through the chamber at 6 l./min. by a Starling Ideal pump (C. F. Palmer). Ether vapour was added by passing the air through a calibrated E.M.O. Inhaler (Pentland Instrument) as described by Epstein and Macintosh (1956). The lower part of the inhaler was immersed in a water bath at 20°. With a flow of 6 l./min., the concentration of ether in the chamber could be changed by about 97% in 10 min., and this time was always allowed for equilibration after each change of ether concentration. All animals were kept in a warm chamber (30 to 35°) for not less than 30 min. before transfer to the observation chamber for exposure to ether. They were exposed to ether, either singly or in batches, and were not restrained. The chamber was closed by a perspex lid in which there were two holes 4 in. in diameter. One was covered by a soft rubber sheet with a slit, through which animals could be introduced or removed from the chamber. The rubber sheet minimized mixing of atmospheric air with the contents of the chamber. When not in use this hole was closed by a perspex shutter. The second hole had a long-sleeved rubber glove attached to its margin so that, when an animal began to stagger, the operator, with the hand inside the glove, could turn it on its back. The time was recorded at which the animal lost its righting reflex. The animal was regarded as being anaesthetized when it no longer responded to pinching of the hind paw.

Sleeping time was the interval during which the righting reflex was absent. Sleeping animals were kept at constant temperature. Deaths were recorded at 24 hr.

Sodium pentobarbitone B.P. (Abbott) was dissolved in distilled water not more than 2 hr. before use. Urethane (ethyl carbamate: M.P. 48 to 50°) solutions were prepared from B.D.H. laboratory reagent in distilled water. Injections of pentobarbitone or urethane were given intraperitoneally with the dose per kg. contained in 10 ml. fluid.

RESULTS

Pentobarbitone

Preliminary experiments are summarized in Table I, which shows that pentobarbitone was more toxic to new-born rabbits and rats than to adults. For example, a dose of 35 mg./kg. of pentobarbitone commonly given to adult rabbits to produce

light anaesthesia of about 1 hr. with subsequent recovery killed 5 of 6 rabbits less than 1 day old (Table I). These animals did not become anaesthetized until they breathed less than twice per minute and were cyanosed.

TABLE I

TOXICITY OF PENTOBARBITONE TO DAY-OLD RABBITS AND RATS

The drug was given intraperitoneally. Environmental temperature, 32°.

Dose (mg./kg.)	Deaths at 24 hr.
Rabbits 1 day old	
25	1/5
28	0/7
32	0/8
35	5/6
Rats 1 day old	
45	4/6
50	3/6
55	6/6
60	5/6
Adult rats	
60	3/7
100	4/7
140	6/7

The sleeping time of new-born rabbits which had received a given dose of pentobarbitone was much greater than that of adolescent or adult rabbits which had received the same dose per kg. (Table II).

TABLE II

THE SLEEPING TIMES OF RABBITS AFTER PENTOBARBITONE

The numerals in parentheses indicate the number of animals. Environmental temperature, 32°.

Dose (mg./kg.)	Range of Sleeping Times (min.)		
	1 Day Old	30–31 Days Old	Adult
6	49–56 (4)		
12	54–143 (4)	8–23 (3)	0 (3)
18	173–265 (4)	0–31 (3)	12–27 (3)
24	300 or more (4)	5–47 (3)	8–72 (2)

Urethane

The sleeping times of both new-born rats and rabbits treated with 400 and 600 mg./kg. of urethane was longer than those of adults (Table III). However, there was a pronounced difference between the depth of anaesthesia produced in young and in old rabbits and in rats with this drug. Only 1 of 4 rabbits, which had received 1.2 mg./kg. of urethane, less than 3 days old did not resist restraint on its back, whereas 4 of 4 adult rabbits which had received the same dose could be restrained on their backs. Young rats reacted to 1.2 g./kg. of urethane similarly to young rabbits.

TABLE III

SLEEPING TIME OF RABBITS AND RATS AFTER URETHANE INTRAPERITONEALLY

The sleeping time is given ± standard error. The numerals in parentheses indicate the number of animals used in each estimate. Environmental temperature 34°.

Dose (mg./kg.)	Sleeping Time (min.)	
	One Day Old	>90 Days
Rabbits		
400	68±13·1 (7)	15±4·5 (8)
600	90± 9·5 (7)	41±8·5 (8)
	Four Days Old	Adult
Rats		
400	28±5·4 (9)	0 (9)
600	43±2·7 (9)	0 (9)

Ether

A group of 54 rabbits of three different ages (1, 3, and 30 days) were exposed to one of three concentrations of ether (4.1, 8.3 or 12.8 vol. %) at one of three temperatures (20°, 30° or 40°). Animals were allocated to experimental groups according to a latin square design and litters were randomized. The interval between exposure to ether and loss of righting reflex became less as the concentration of ether was increased. It was 9 to 11 min. at 4.1 vol. % ether, 2 to 9 min. at 8.3 vol. % and 2 to 4 min. at 12.8 vol. %. This interval was affected neither by the temperature nor by the age of the rabbits.

There was a difference in the time taken for rabbits of different ages to regain their righting reflex after removal from ether (Table IV). Two groups each of 5 rabbits (4 days old and 28 to 32 days old) were simultaneously exposed to 12.8 vol. % ether at 32° for 3.75 min. At the end of this time all rabbits had lost their righting reflex. The concentration of ether was then reduced to 1.9 vol. % and the time taken to regain the righting reflex was observed. This experiment was repeated on a different day, and, in spite of divergence of the mean times, all older rabbits

TABLE IV

TIME TAKEN BY RABBITS TO REGAIN RIGHTING REFLEX WHEN EXPOSED TO 1·9 VOL. % OF ETHER AFTER EXPOSURE FOR 3·75 MIN. TO 12·8 VOL. % OF ETHER

Environmental temperature, 32°. The times are means ± standard error.

	Time (sec.)	
Age:	<4 days	28–32 days
Exp. No. 1 ..	> 60	30±3·8
,, ,, 2 ..	>180	69±6·0

regained their righting reflexes before any of the younger ones.

Two rats of different ages were placed simultaneously in an ether-air mixture at 30°. Five such pairs of rats were observed at each of three different concentrations of ether between 10.2 and 17.5 vol. %. Table V shows that there was little

TABLE V

TIME TAKEN BY RATS TO LOSE RIGHTING REFLEX AND TO BECOME ANAESTHETIZED WHEN EXPOSED TO ETHER.

Environmental temperature, 30°. There were 5 animals in each group. The times in min. are means ± standard error.

Ether (Vol. %)	Time to Lose Righting Reflex		Time to Lose Response to Pinch	
	5 Days	24–28 Days	5 Days	24–28 Days
10·2	65±5·7	63±2·0	625±38	239±41
14·2	52±3·5	47±2·2	435±26	131±12
17·5	42±4·6	45±1·6	463±26	125± 9

difference between the times at which rats of different ages lost their righting reflex, but the younger rats took considerably longer before they became anaesthetized. The concentrations of ether used in these experiments can be regarded as high.

In other experiments, litters of rats of different ages were exposed simultaneously at 32° to lower concentrations of ether, either 1.9 or 4.1 vol. %. Table VI shows that all rats less than 1 day old had lost their righting reflex after 180 min. of exposure to 1.9 vol. % ether, whereas none of the older rats had lost their righting reflex after 240 min. of exposure to the same concentration of ether. In 4.1 vol. % ether, all the younger rats had lost their

TABLE VI

LOSS OF THE RIGHTING REFLEX IN NEW-BORN AND ADULT RATS EXPOSED TO ETHER

Environmental temperature, 32°.

Ether (Vol. %)	Age	No. of Rats in Group	Number of Rats Without Righting Reflex after Exposure for (Min.)							
			10	20	30	60	90	120	180	240
1·9	<1 day	16	0	3	1	6	10	10	16	16
	9 weeks	17	0	0	0	0	0	0	0	0
4·1	4 days	11	11	11	11	11				
	4 weeks	6	0	5	6	6				
	9 ,,	5	0	1	5	5				

righting reflex after 10 min., but the older ones did not lose their righting reflex until after 20 min.

As with rabbits, the older rats also regained their righting reflex more quickly after ether. Groups of rats of several ages were exposed to 12.8 vol. % ether for 2 min., by which time all animals had lost their righting reflex. The concentration of ether was then reduced to 1.9 vol. % and the rats were examined after a further 20 min. By this time all the adults and a few of the older rats had regained their righting reflex, but none of the younger rats had righted themselves. These experiments were carried out at 20°, 30° or 40°, but, as the temperature did not appear to influence either the time to lose or to regain the righting reflex, the results have been combined in Table VII.

TABLE VII

DURATION OF LOSS OF THE RIGHTING REFLEX IN RATS EXPOSED TO 12·8 VOL. % OF ETHER FOR 2 MIN. AND THEN TO 1·9 VOL. % OF ETHER FOR 20 MIN.

For further explanation, see text.

Age (Days)	Number of Rats without Righting Reflex after 20 Min.
0–5	46/46
5–10	31/31
15–20	9/9
25–30	9/9
35–40	14/20
90–95	3/20
Adult	0/7

DISCUSSION

It is evident from these results that new-born rats and rabbits differ from adults in their reactions to anaesthetic drugs, but these differences are not

consistent. After small doses of urethane (400 or 600 mg./kg.) new-born rats and rabbits lose their righting reflex for longer than adults ; whereas a dose of urethane, which in both adult rats and rabbits produced relaxation sufficient for surgical manipulations, did not relax or anaesthetize new-born rats and rabbits. Similarly with ether, new-born rats did not become anaesthetized so quickly as adult rats although the new-born rats and rabbits lost their righting reflex as soon as or sooner than the adult animals and could be maintained, without their regaining a righting reflex, at lower concentrations of ether than could adult rats. Pentobarbitone, which produced longer loss of righting reflex in new-born rabbits than in adults, did not produce anaesthesia in new-born rabbits until the respiration was grossly depressed, and was more toxic to new-born rats and rabbits than to adults.

The finding that new-born rabbits were not anaesthetized by a dose of urethane sufficient to anaesthetize an adult rabbit has also been observed by Adamsons (personal communication) and by Downing (personal communication), both of whom used urethane to anaesthetize rabbits of different ages for surgical procedures (Adamsons, 1959 ; Downing, 1960). However, Cross, Dawes and Mott (1959) found that about 1/4 of the dose of chloralose required for an adult sheep was sufficient to produce light surgical anaesthesia in a new-born lamb.

The prolonged action of barbiturates in new-born mice has been described by Jondorf *et al.* (1959). These authors also found that all of an injected dose of hexobarbitone was present in mice less than 24 hr. old 3 hr. after injection, whereas in 21-day-old mice only 70% of the dose remained after 3 hr. The metabolism of amidopyrine and phenacetin was similarly reduced in new-born mice. The side chain oxidation of hexobarbitone by liver homogenates from rabbits 2 to 3 weeks old was only 5 to 37% of that from adult rabbits (Fouts and Adamson, 1959) : while new-born guinea-pig tissues were unable to form glycuronides (Brown and Zuelzer, 1958). Indeed there is much evidence that the concentrations of various enzymes in mammals are low at birth and increase during the first days of life (Potter, Schneider and Liebl, 1945 ; Reem and Kretchmer, 1957 ; Dawkins, 1959).

It is attractive to argue that the differences between the effects of urethane, pentobarbitone and ether in new-born and adult animals are due to functional differences in the central nervous systems. However, although there are known differences in enzyme concentrations in various parts of the brain at different ages, there is not sufficient known of the mechanism of action or of the distribution of the anaesthetic drugs compared in this paper to say how the observed differences occur. On the other hand it is probable that the prolonged action of pentobarbitone is due in part to the slow systemic breakdown of the drug by new-born animals. The same may be true of urethane, 90% of which in normal adult mice is metabolized in 24 hr. (Mitchell, Hutchison, Skipper, and Bryan, 1949). Ether is not metabolized to any appreciable extent but is eliminated through the breath and excreta (Haggard, 1924) so that slow metabolism of ether cannot explain its prolonged action in new-born animals.

The differences in the effects of these three anaesthetic drugs on new-born animals show that it is not possible to generalize about the greater " sensitivity to anaesthetics " of new-born animals as compared with adults, and with our present knowledge it is not possible to predict reliably how a new type of anaesthetic drug will act in a new-born animal.

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EXPERIMENTS ON THE PHARMACOLOGY OF HYDROXYDIONE SODIUM SUCCINATE

BY

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The actions of hydroxydione sodium succinate (" Viadril ") have been studied in chloralose or decerebrate cats, or cats otherwise untreated. It is virtually devoid of action at the neuromuscular, ganglionic, or peripheral adrenergic synapse. After rapid intravenous injection, hydroxydione produced a transient hypotension with little bradycardia and stimulated the respiration. These effects were over in 1 to 2 min. and were reduced if injection was slow or the hydroxydione diluted. Vagotomy did not alter the depressor response, but reduced the respiratory stimulation. In the untreated or the decerebrate animal, as much as five times the anaesthetic dose of hydroxydione was required to depress the respiration or blood pressure. Chloralose potentiated these depressant actions 5 to 10 fold. Both the circulatory and the respiratory depression appeared to be mediated centrally. Decerebrate rigidity was readily lessened by hydroxydione, but the pinna reflex and conjunctival reflex were relatively resistant to its action, and the knee jerk outstandingly so.

Fine transient muscular fasciculations, not unlike those produced by suxamethonium, have occasionally been observed by one of us during hydroxydione administration in man, occurring shortly after the onset of unconsciousness. They appear first in the muscles of the face, spread to the upper limbs and trunk, and die away after a few seconds. Such an observation suggested that hydroxydione might have some neuromuscular action. The possibility became more important in the light of the relaxation which hydroxydione anaesthesia can produce, especially in the pharynx and the larynx. The experiments described below started, therefore, with an investigation, in cats under chloralose, of the neuromuscular action of the drug. It was found, however, that even large doses of hydroxydione had no neuromuscular action. But in the animal under chloralose a somewhat unexpected depression of the respiration by hydroxydione was observed which prompted a fuller investigation of its pharmacology. One interesting finding has been the great potentiation of the respiratory depressant action of hydroxydione by the presence of chloralose.

METHODS

Cats were used for most experiments. After induction with ethylchloride and ether, anaesthesia was maintained with chloralose 60 to 80 mg./kg. The blood pressure was recorded by a siliconed, heparinized cannula in one carotid artery. The respiration was recorded, after a tracheal cannula had been inserted, by the aid of respiratory valves and a respiration recorder on the expiratory side (Paton, 1949). When required, the twitches of the tibialis anterior muscle were recorded on the smoked drum with a steel spring myograph, in response to supramaximal excitation of the sciatic nerve through shielded electrodes in the thigh. Rectangular pulses of 0.5 m.sec. duration were delivered at varied frequencies, usually one every 10 sec. Action on an autonomic ganglion was tested by recording the contractions of the nictitating membrane with a lever of magnification about 5-fold and load about 2 g. writing frontally on the smoked drum, the membrane being excited to contraction by supramaximal preganglionic shocks through the cervical sympathetic nerve in the neck which had previously been separated from the vagus and divided from its central connexions. The contents of the eyeball were removed. Rectangular pulses of 0.5 m.sec. duration were applied, at 10 shocks per sec. either for a period of 15 sec. every min. or continuously, through platinum electrodes. To make injections a venous cannula was inserted into the femoral or jugular veins, and all injections were washed in with 2 ml. saline.

In some experiments decerebration was performed; a trephine hole was made in the parietal bone and, after incision of the dura, a transection made between the inferior and superior colliculi towards the pituitary fossa; the hemispheres were then removed. To reduce bleeding during the operation both carotid arteries were tied and a vertebral clamp adjusted to control bleeding as necessary. The animal was left for 0.5 hr. to 1 hr. before further dissection so that satisfactory clotting of the cerebral vessels could occur.

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In two cats induction of anaesthesia was performed with hydroxydione, the drug being made up in 1% procaine, and injected into a vein on the foreleg. When full anaesthesia had been produced one or more of the procedures just mentioned were used.

The doses of drugs are given in terms of their salts. These were hydroxydione sodium succinate (" Viadril," Pfizer), adrenaline tartrate, noradrenaline tartrate, acetylcholine chloride, histamine acid phosphate, succinylcholine di-iodide, *d*-tubocurarine chloride. The hydroxydione was normally made up as a 10% solution either in saline or, as described by Galley and Lerman (1959), in 0.25% or 0.5% procaine. The procaine made no detectable difference to the effects seen.

Results

Actions on the Neuromuscular Junction

In the cat under chloralose a dose of hydroxydione (5 to 10 mg./kg.), which can produce severe respiratory depression, leaves the twitch of the tibialis in response to excitation of its motor nerve completely unaffected (Fig. 1, *a* and *b*). There are neither

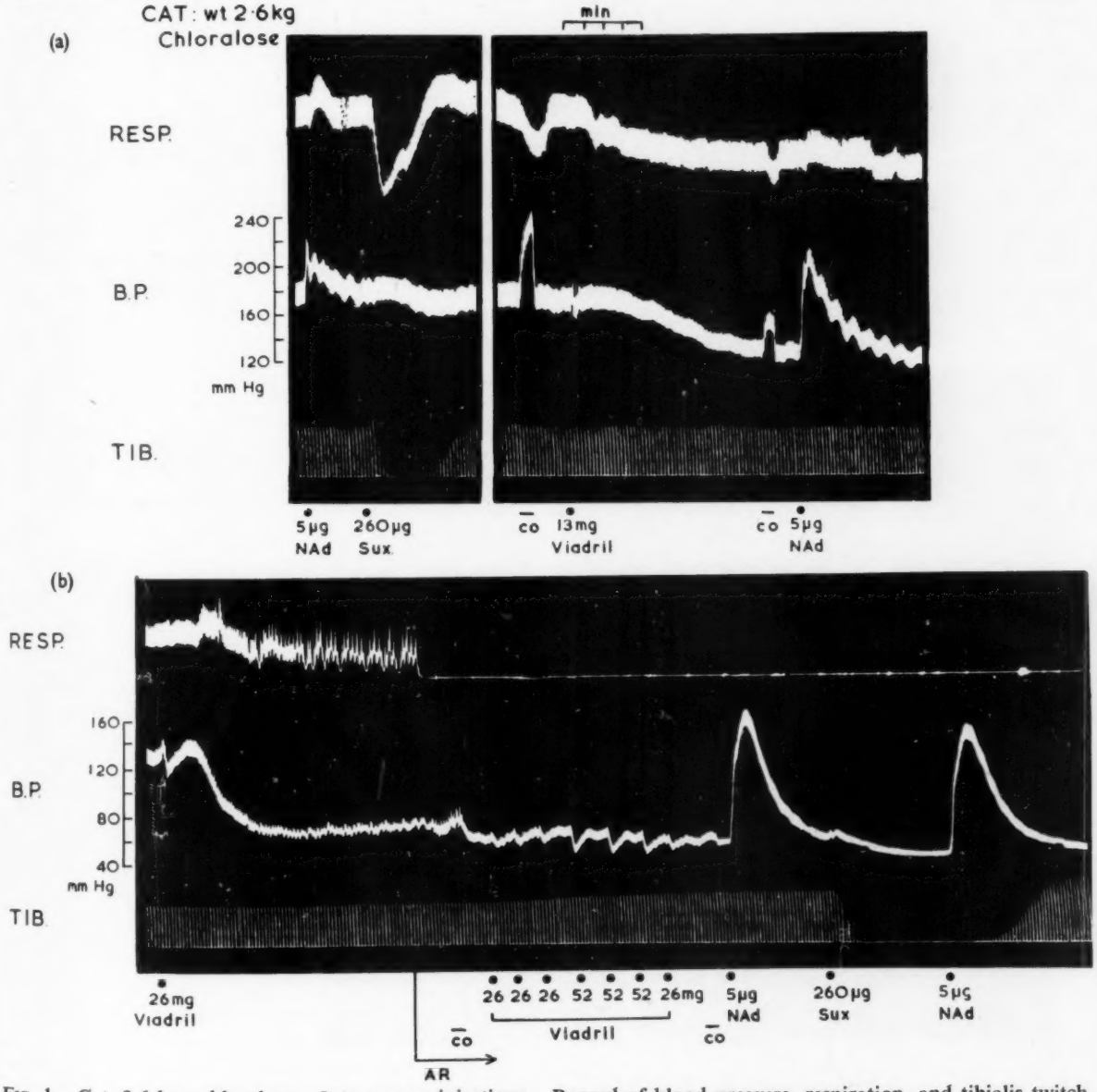

FIG. 1.—Cat, 2.6 kg., chloralose. Intravenous injections. Record of blood pressure, respiration, and tibialis twitch. CO, bilateral carotid occlusion for 30 sec. AR, artificial respiration. NAd, noradrenaline. Sux, suxamethonium.

preliminary fasciculations to be seen nor potentiation of the twitch nor any sign of neuromuscular block. Even doses up to 100 mg./kg. (Fig. 1*b*), which produce profound circulatory effects in the cat under chloralose, are either devoid of any effect on the muscle twitch or even slightly potentiate it. In addition, a test was made of the interaction of hydroxydione with succinylcholine and *d*-tubocurarine. Succinylcholine given to a cat which was under the influence of 10 mg./kg. hydroxydione did not vary from its usual behaviour; and a dose of 20 mg./kg. hydroxydione during recovery from succinylcholine paralysis did not influence the course of recovery in any way. After a massive dose of hydroxydione (100 mg./kg.) the fasciculations normally seen were abolished (Fig. 1*b*), and the neuromuscular block produced came on a little more slowly and was somewhat prolonged. If so small a change in the action of suxamethonium is produced by a dose 5 to 10 times the normal human anaesthetic dose, any such effect must be negligible in practice. With tubocurarine, corresponding observations were made; after 100 mg./kg. hydroxydione, it was possibly a little potentiated, and had a more prolonged action. These observations imply that, in a very large dose, hydroxydione has a slight ability to lessen specific depolarization of the endplate, a property shared, in much more intense form, by many anaesthetics.

Supporting evidence for the conclusion that hydroxydione had virtually no neuromuscular action was obtained indirectly in another way. In the decerebrate animal after total doses of hydroxydione up to 230 mg./kg., and with a dose of 500 mg./kg. after induction with hydroxydione, the knee jerk could still be elicited. The presence of a knee jerk necessitates intact neuromuscular transmission; this result is an even more cogent reason for rejecting any significant neuromuscular action.

Action on Autonomic Ganglia

Fig. 2 shows the effect of 10 mg./kg. hydroxydione on the response of the nictitating membrane to preganglionic excitation, a dose which produced the usual substantial sustained hypotension seen in chloralosed cats. Immediately after the injection, there was possibly a slight weakening of the response of the membrane to continued stimulation; and about 10 min. later, when the hypotension was fully developed, there was a doubtful reduction of the

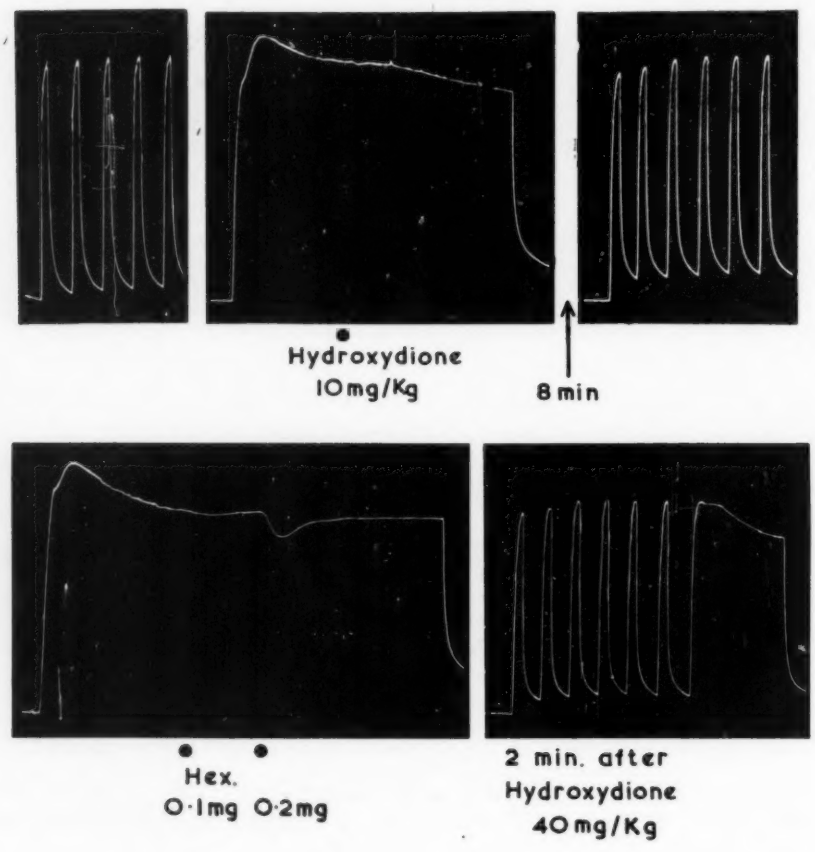

FIG. 2. — Cat, chloralose. Intravenous injections. Response of nictitating membrane to preganglionic excitation at 10/sec., either for 15 sec. every min., or continuously.

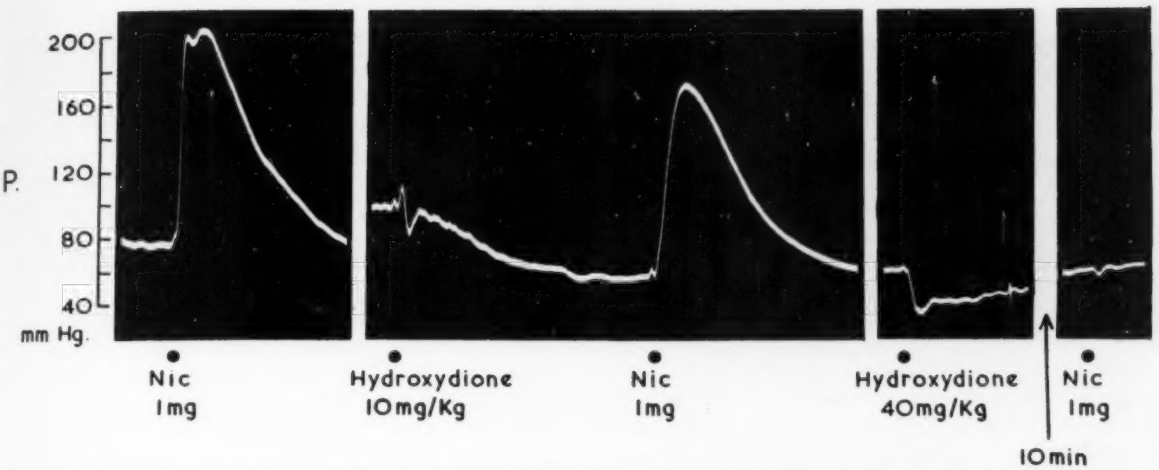

FIG. 3.—Cat, chloralose. Record of carotid arterial blood pressure. Artificial respiration. Responses to nicotine (Nic) before and after 10 and 40 mg./kg. hydroxydione.

response to intermittent excitation. With a higher dose, of 40 mg./kg., the response to intermittent stimulation was definitely reduced; and with continued excitation, the response was not quite as well maintained as usual. Hydroxydione thus has a slight ganglionic action, around 1/300 of the potency of hexamethonium, detectable but not sufficient to account for the hypotensive action.

A test was also made of the effect of hydroxydione on the pressor response to nicotine (Fig. 3). At 10 mg./kg., the rise in blood pressure was hardly altered, although its time course was modified slightly. With 40 mg./kg., however, the action of 1 mg. of nicotine was abolished. This anti-nicotine action is more pronounced than would be expected from the effect of hydroxydione on ganglionic transmission; but it must be remembered that nicotine, in addition to exciting autonomic ganglia, also stimulates chemoreceptors and exerts other central actions.

The fact that the nictitating membrane response to preganglionic excitation was little altered by hydroxydione also implies that hydroxydione has a negligible action at the junction between the sympathetic nerves and smooth muscle, and on the responses of smooth muscle to the sympathetic transmitter. The latter point was tested directly. A large dose of hydroxydione, indeed, increases the rise in blood pressure produced by noradrenaline (Fig. 1*b*), presumably because the blood pressure is lower and the buffer mechanisms paralysed. In short, hydroxydione has little peripheral synaptic effect, whether tested on the sympathetic effector junction, the autonomic ganglion or the neuromuscular junction.

Effects of Hydroxydione on Respiration and Blood Pressure

Animals under Chloralose.—In our first experiment, on a cat under chloralose, the somewhat startling observation was made that a dose of 10 mg./kg. of hydroxydione abolished the respiration in between 3 and 4 min. This is quite out of keeping with clinical experience (Lerman, 1956), so that a further analysis was made. In the chloralosed animal this dose always depressed respiration strongly, at least 40%. Even 5 mg./kg. had a distinct effect on respiration (Fig. 1*a*). Usually 20 mg./kg. would produce apnoea, but sometimes a higher dose was needed.

The immediate effect of hydroxydione on the respiration is usually a transient stimulation, occurring soon after the injection, increasing the minute volume sometimes as much as two-fold, sometimes only by 20 to 30%, with a rapid return to normal. It is after this that the depression of the minute volume occurs, accompanied by some slowing of respiration. Even with a large dose of hydroxydione there is some delay before respiratory paralysis ensues; and it probably cannot be produced in a shorter time than 2 or 3 min. The respiratory minute volume recovers again with time; after 10 to 20 mg./kg., recovery takes 30 to 60 min. During a reduction of respiration by hydroxydione, provided asphyxia is not produced, the minute volume often becomes more regular; spontaneous fluctuations, or reflex responses to rise or fall of blood pressure due to adrenaline or histamine or to stimulation of the cervical sympathetic trunk, are reduced. Thus, even in the experiment of Fig. 1*a* where the respiration was

already unusually stable, the respiratory effects of carotid occlusion and of 5 μg. noradrenaline were reduced by 10 mg./kg. hydroxydione.

Accompanying the respiratory action is a somewhat complicated response of the blood pressure. First, with a dose of 10 mg./kg. or more, is a pronounced transient fall in blood pressure; this may be as much as 50 mm. of Hg or more, according to the dose and speed of injection. It has a very short latency after the injection, and the blood pressure returns to the normal level in about 30 sec.

After this initial hypotension a second, pressor response up to 40 mm. and lasting 2 to 5 min. may be seen. There is no noticeable tachycardia during this time. The rise in blood pressure appears to occur only when there is a reduction of minute volume; but it is sometimes absent even when the respiration is depressed. This phase then passes into the third and main response, a slowly progressing hypotension which may reach its lowest point up to 15 or 20 min. after the injection; the blood pressure then slowly rises again, to return to normal at a time depending on the dose given.

The final prolonged hypotension appears to depend on a central vasomotor depression. The experiments on autonomic ganglia, mentioned earlier, exclude a peripheral paralysis of sympathetic effector organs as the cause, and this was confirmed by verifying that, for instance, the action of adrenaline and noradrenaline was unimpaired during the hypotension. (The depressor effects of histamine, and of histamine liberation, are also still demonstrable, although reduced as is usual when the blood pressure falls.) The hypotension is not accompanied by any marked bradycardia, and vagotomy did not influence it. The most reasonable explanation for it, therefore, is that it is due simply to reduction of central vasomotor tone. This conclusion was substantiated by finding that during the hypotension due to hydroxydione, the rise in blood pressure elicited reflexly by bilateral carotid occlusion was impaired despite the continuing effectiveness of autonomic transmission and of noradrenaline (Fig. 1*a*). With 5 mg./kg. hydroxydione (lowering the blood pressure by about 40 mm.) a considerable reduction of the carotid occlusion response occurred; with 10 mg./kg. (lowering the blood pressure to 60 mm.) it was virtually abolished (Fig. 1*b*) although 5 μg. noradrenaline had a larger pressor effect than normal. Three hours later the carotid occlusion response had recovered considerably; the blood pressure was still low, but, after 7 hr. anaesthesia, this is of doubtful significance.

Decerebrate Animals

If the animal is decerebrated under ether, and an interval of 1 hr. or more allowed after ceasing ether administration, one can test hydroxydione on the respiration and circulation in the virtual absence of any other anaesthetic. When this is done, the initial respiratory stimulant and depressor actions are still present, with the same time course; the respiratory stimulation, indeed, is more pronounced than in the presence of chloralose. But the subsequent depression of the respiration is absent, until relatively enormous doses of hydroxydione are given. Similarly, the blood pressure, after the initial hypotension, returns permanently to its original level, and there is little sign either of the pressor effect or of the slow prolonged hypotension. Thus, in one animal, 140 mg./kg. hydroxydione given in divided doses over a period of 40 min. had nothing but a stimulant action on the respiration. In another, a single dose of 40 mg./kg., following previous divided dosage of 70 mg./kg. (itself ineffective), only stimulated respiration. In a third animal, it required 40 mg./kg. to depress minute volume by 25%. The blood pressure responses are harder to interpret, since the pressure tends to decline from its initial level in any case; but it appeared that no persistent hypotension occurred with doses under 40 mg./kg. and in one experiment 110 mg./kg. failed to reduce the blood pressure.

The decerebrate animal appeared, in short, to be something like 5 to 10 times more resistant than the chloralosed animal to the depressant effect of hydroxydione on respiration and blood pressure. So large a difference raised the question whether decerebration had removed some nervous structure necessary for the action of hydroxydione. This was tested by giving chloralose to three decerebrate animals. In one, which had received 140 mg./kg. hydroxydione with no depression of respiration, 40 mg./kg. chloralose (half the standard dose) produced apnoea in 3.5 min. In the other two, 60 mg./kg. chloralose itself depressed the respiration considerably; after a period of recovery, 10 mg./kg. hydroxydione was now itself able to produce apnoea. There was thus a powerful synergism between the two drugs in the decerebrate animal, each potentiating the depressant action of the other; it was of such a magnitude that a dose of hydroxydione, a small fraction of that necessary by itself to exert respiratory depressant action, combined with a dose of chloralose much smaller than normally employed, could cause complete abolition of respiratory movement. The fact that giving chloralose to the decerebrate cat allowed the depressant action of hydroxydione to be reproduced as in the intact cat under chloralose makes it

unnecessary to suppose that decerebration removes some nervous structure requisite for the action of hydroxydione.

The opportunity was also taken to make some qualitative observations in these decerebrate animals on muscle tone, the knee jerk, the conjunctival reflex and the pinna reflex. Decebrate rigidity, and the clonic character of the response to tapping the quadriceps tendon, were obviously reduced by doses of 20 to 30 mg./kg. hydroxydione; but it proved generally impossible to abolish the reflexes, although the conjunctival reflex was weakened by large doses. A similar observation had been made in chloralosed animals: that even when apnoea was produced with hydroxydione, the knee jerk remained.

Induction of Anaesthesia with Hydroxydione

Having found that hydroxydione in the decerebrate cat appeared to be a remarkably safe anaesthetic, an induction of anaesthesia with hydroxydione alone was performed. In one animal a dose of 10 mg./kg. intravenously sedated the animal, and a further 10 mg./kg. 20 min. later deepened the sedation, and after another 20 min. interval a further 20 mg./kg. produced surgical anaesthesia. In another, approximately 80 mg./kg. in divided doses over 45 min. produced a similar effect.

Some of the observations previously made were then repeated (Fig. 4). Under these conditions, a dose of 10 or 20 mg./kg. hydroxydione had little or no depressant effect on respiration, and did not cause any prolonged hypotension. The usual transient hypotension, with a marked accompanying respiratory stimulation at the time of injection, was still seen. The promptness and transience of these two responses suggest that they are due to the presence of a high concentration of hydroxydione in the blood during its first circulation after the injection. This was confirmed by finding that both responses are much attenuated, either by making the injection slowly, or by diluting the injected solution ten-fold (Fig. 4*a*). Our first impression was that the hyperpnoea was secondary to the hypotension: for a similar phenomenon is often seen with depressor drugs. But, recorded on a fast-moving drum, the respiratory response sometimes precedes, by a few seconds, the hypotension, so that the latter can hardly be the sole cause of the respiratory change.

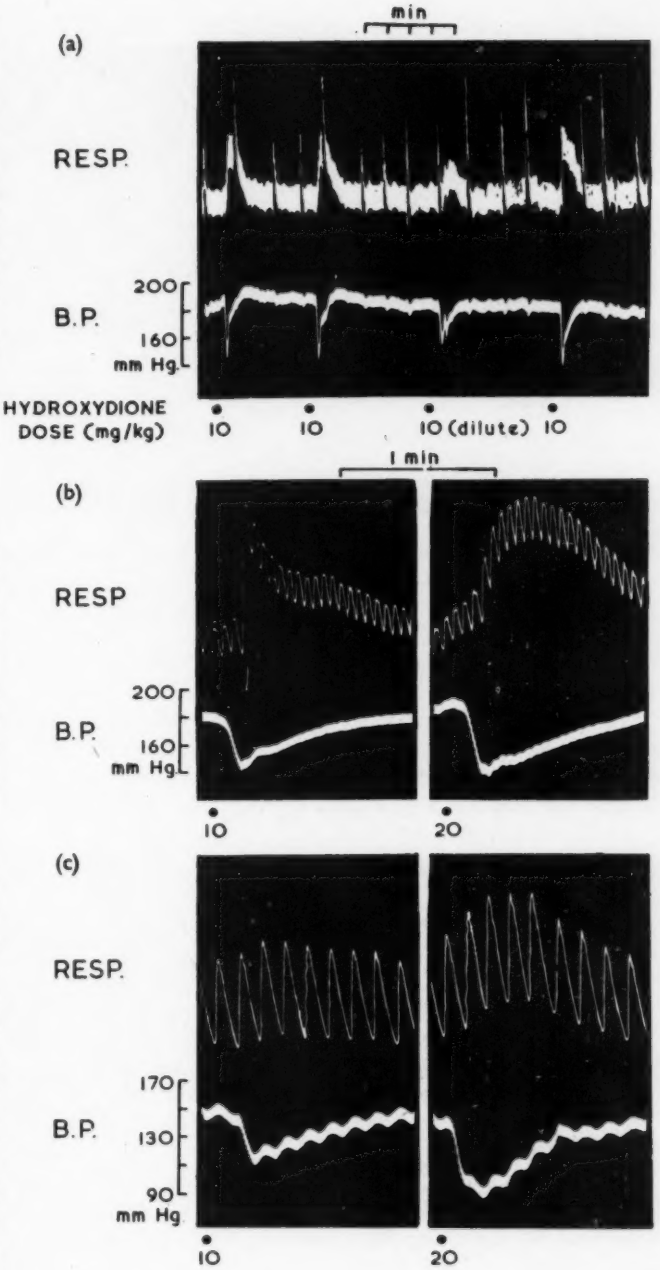

FIG. 4.—Cat, hydroxydione anaesthesia. Intravenous injections. Record of respiration and blood pressure. (*a*) Responses to hydroxydione 10 mg./kg. given in 0.23 ml. washed in with 2 ml. saline, except third injection in 2.3 ml. with 2 ml. washed in. (*b*) Responses to 10 and 20 mg./kg. hydroxydione on a 10 times faster drum. (*c*) Responses to 10 and 20 mg./kg. hydroxydione after cutting both vagi.

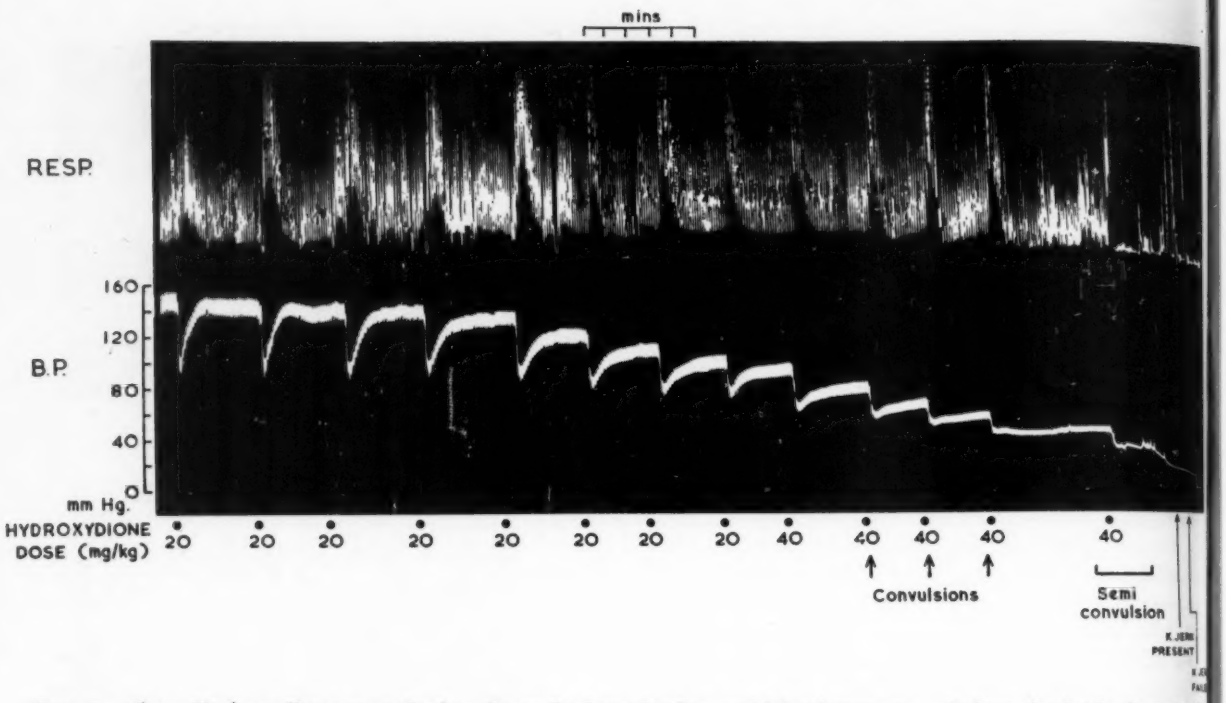

FIG. 5.—Cat. Hydroxydione anaesthesia. Record of respiration and blood pressure. Before the beginning of the record, 240 mg./kg. hydroxydione had been given over 2 hr. 40 min.

This analysis was confirmed by sectioning the vagi. As a result the hypotension produced by hydroxydione was reduced slightly, but its time course and the slight slowing of heart rate during the fall of pressure were unchanged. The respiration was more affected; the stimulant effect was much reduced; and in one experiment in which the first effect of hydroxydione had been to suppress one or two breaths before the stimulation of respiration, this transient apnoea disappeared. It appears, therefore, that the vagi play some part in the initial respiratory response to hydroxydione.

The experiment of Fig. 4 was terminated by giving repeated doses of hydroxydione at 5 min. intervals, to examine the response to massive dosage (Fig. 5). After around 320 mg./kg., the blood pressure began to fail to return to its original level after each injection. After 480 mg./kg., each injection gave rise to a species of transient convulsion, which, after the last dose (total 600 mg./kg.), was more persistent and was at last accompanied by respiratory failure. It was remarkable that the knee-jerk could still be elicited, and not until effective respiration had ceased for several min. did it disappear.

DISCUSSION

Hydroxydione deserves attention as representing a type of anaesthetic structurally quite distinct from those in general use. Although, as a rule, the chemical structure of the general anaesthetics is less important than their physical properties, it may well be that the steroid nature of hydroxydione is responsible for some of its novel features. In discussing these, comparisons with other anaesthetics will be made.

As with other drugs given intravenously, the concentration in the blood is very high during the first one, possibly two, circulation times after the injection. Gray and Paton (1949) showed that, in the cat, an injection of volume about 3 ml. was only diluted about ten-fold in the blood in its first passage from a femoral vein to carotid artery; and that the injection " slug " was still detectable during the second circulation. We have concluded that the first response to hydroxydione, a fall in blood pressure and a stimulation of respiration, is due to this " slug effect "; for the time relations are correct, and this first response is greatly reduced by diluting the injected solution or by slow injection. The hypotension is accompanied by only a slight bradycardia and survives vagotomy, so that it is

not of the Bezold-Jarisch type. The respiratory response, primarily stimulant but occasionally preceded by one or two " dropped breaths," appears to depend in part on vagal pathways; the remainder may well be secondary to the hypotension. We have not analysed this initial response further, but it is not altogether a surprising consequence of the sudden exposure of the heart and lungs to a concentration of around 10^{-3} hydroxydione. It is of little significance for clinical practice, where a much greater dilution of the injected material will occur. It compares not unfavourably with thiopentone, with which immediate cardiac depression may be severe, and accompanied by apnoea.

The subsequent responses of the blood pressure and respiration depend on the intensity of the depression of vasomotor and respiratory centres. In the animal under chloralose, after a latency of a few minutes a slowing of respiration and reduction of tidal air lead to a fall of minute volume. There is a fall of blood pressure usually of similar time course, although sometimes the blood pressure may rise initially for a few min. during the early stages of the respiratory depression. Both respiratory and blood pressure depression reach their peak in 10 to 20 min. At this stage, spontaneous fluctuations or changes of reflex origin are reduced or abolished, although the responsiveness of the blood vessels to directly acting drugs (adrenaline, noradrenaline, histamine) is retained. Recovery is gradual; the effect of a dose of 10 to 20 mg./kg. lasts 30 to 60 min.

Although the reactions of the animal under chloralose allow the type and duration of action of hydroxydione to be studied conveniently, they give an erroneous picture of their magnitude. In the decerebrate or normal animal, it was not possible to produce apnoea with doses less than 140 mg./kg., and hypotension was equally slight; but under chloralose, 5 mg./kg. both reduced blood pressure and minute volume substantially. The mutual potentiation between chloralose and hydroxydione is an interesting phenomenon, and may well extend to other anaesthetics; Lerman (1956) has already commented on a potentiation by hydroxydione of nitrous oxide. More important for practice, however, is an estimate of the safety of hydroxydione, expressed as the ratio of respiratory or circulatory depressant dose to anaesthetic dose. We have not made the systematic study necessary for this; but it is clear, from our own result (that roughly 50 mg./kg. will produce surgical anaesthesia) in conjunction with results reported elsewhere, that the safety factor is of the order of 5-fold, possibly higher. Some additional observations, which we have not described in detail, are relevant here. If apnoea is produced with hydroxydione, and allowed to continue for about 3 min. until circulatory failure occurs, it only requires artificial respiration and, if necessary, cardiac massage to restart quite a vigorous heart beat. At no time have we seen cardiac irregularities, even under such stringent conditions. It appears, in short, to be comparable with, or superior to, ether in its safety.

Finally, the lack of peripheral synaptic action is of interest. Ether is known to have quite a pronounced " curariform " action, shared in more or less degree by the other volatile anaesthetics. The barbiturates also have neuromuscular actions, and a power to block ganglia, which, with amylobarbitone, formed the basis of a test for neurogenic hypertension.

Hydroxydione thus presents features of considerable interest. It appears to be both safe and specific. Its time course of action differs from, say, thiopentone, but this merely means it must be used differently. If its principal disadvantage in practice (that of surface-activity, on which its local irritant effect in the tissues appears to depend) could be overcome, it, or similar compounds, would represent very valuable anaesthetic agents.

We are much indebted to Dr. H. R. Reinert, of Pfizer, for supplies of Viadril; and to Mr. D. A. Green and his staff for technical assistance.

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PERIPHERAL VASCULAR EFFECTS OF BRETYLIUM TOSYLATE IN MAN

BY

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After intra-arterial infusion of bretylium tosylate (12.5 mg.), the reflex changes in vasoconstrictor tone which normally occur in the forearm with body cooling, positive pressure breathing, the Valsalva manœuvre and postural change were greatly reduced or abolished. Reflex vasodilatation mediated by cholinergic fibres in response to body heating or to emotional stress was little affected. It was concluded that bretylium can selectively block the activity of sympathetic noradrenergic fibres without causing a similar block of sympathetic cholinergic fibres. As the responses to intravenous or intra-arterial infusions of adrenaline or noradrenaline were not reduced after bretylium, it was concluded that bretylium interferes with the activity of noradrenergic fibres rather than with the activity of the noradrenaline released at the nerve ending. After bretylium infusion, forearm and hand blood flow did not often rise to levels characteristic of full release of vasoconstrictor tone. As infusion of bretylium into a nerve-blocked forearm resulted in a pronounced reduction in flow, it is concluded that bretylium also has a constrictor effect on blood vessels. The state of the vessels following an infusion of bretylium appears to depend on the balance between this constrictor action and the longer-acting sympathetic blocking effect.

Bretylium tosylate ("Darenthin," Burroughs Wellcome) is one of a series of benzyl quaternary ammonium compounds described by Boura and Green (1959). Experiments in cats suggested that it blocks the peripheral sympathetic (adrenergic) nervous system selectively, without antagonizing the effects of released or injected adrenaline or noradrenaline, and without depression of the parasympathetic or central nervous systems. A preliminary trial in hypertensive patients by Boura, Green, McCoubrey, Laurence, Moulton and Rosenheim (1959) has shown that it lowers the supine blood pressure slightly and causes postural hypotension; and measurements of the circulatory response to the Valsalva manœuvre in a healthy volunteer by Dornhorst (1959) confirmed that the drug produces effects characteristic of block of the sympathetic system.

In the present experiments we have studied the effect of bretylium tosylate on certain vascular reflexes in the human hand and forearm. A brief account of this work has previously been published (Blair, Glover, Kidd and Roddie, 1959).

METHODS

The experiments were carried out on male normotensive subjects, who lay supine on a couch in a laboratory maintained at a temperature of 20 to 21°. The subjects were lightly clad but covered with a blanket. Blood flow in the hands or forearms was measured by venous occlusion plethysmography. A needle was inserted in the left brachial artery in the antecubital fossa, and through it 0.9% (w/v) saline was infused at a rate of 4 ml./min. using a mechanically driven syringe. In most experiments the dose of bretylium was 12.5 mg. infused over a period of 4 min. The preparation of bretylium tosylate contained 1% benzyl alcohol as a preservative. Infusion of the vehicle alone as supplied by the manufacturers did not produce any change in forearm blood flow. In some experiments adrenaline hydrochloride or (−)-noradrenaline bitartrate was infused intravenously (10 μg./min.) or intra-arterially (1 μg./min.) for 4 to 5 min. Ascorbic acid (0·001%) was added to these infusates as a preservative (Gaddum, Peart and Vogt, 1949).

The various stimuli used to elicit vascular reflexes in the forearm or hand were as follows:

Valsalva Manœuvre.—The subject blew into a rubber tube, raising a column of mercury to a height of 50 mm. and maintained this without closing the glottis for 10 sec. This causes an increase in vasoconstrictor tone in skeletal muscle (Sharpey-Schafer, 1955; Roddie, Shepherd and Whelan, 1958).

Emotional Stress.—The subject carried out a mental arithmetic test, a series of additions and subtractions of two or three digit numbers as quickly as possible, with a metronome ticking and with frequent distractions and criticisms. Emotional stress causes excitation of choliner-

gic vasodilator fibres to skeletal muscle (Blair, Glover, Greenfield and Roddie, 1959).

General Body Cooling.—The room temperature was quickly lowered to 16 to 18°. The subject's feet and calves were immersed in a water bath at 17° and as much of the body surface as possible was sponged or sprayed with water at the same temperature. After the first few min. shivering ceased, and the subject was reasonably comfortable during the period in which observations were made. Body cooling causes an increase in vasoconstrictor tone in forearm skin (Roddie, Shepherd, and Whelan, 1957a; Blair, Glover and Roddie, 1959) and in the hand.

General Body Heating.—The feet and calves were immersed in a water bath at 43 to 45° and the subject was wrapped in blankets. Body heating causes reflex vasodilatation in the skin of the forearm and hand. In the hand this is due to release of vasoconstrictor tone (Arnott and Macfie, 1948; Gaskell, 1956; Roddie, Shepherd, and Whelan, 1957b), but in the forearm it is due to an active vasodilator mechanism (Grant and Holling, 1938; Edholm, Fox, and Macpherson, 1957; Roddie *et al.*, 1957a; Fox and Hilton, 1958).

Positive Pressure Breathing.—The subject breathed through a mouthpiece supplied with air at a pressure of 15 mm. Hg above atmospheric for 2 min. This causes reflex vasoconstriction in the forearm (Blair, Glover, and Kidd, 1959).

Single Deep Breath.—The subject carried out a single maximal inspiration and then resumed breathing as

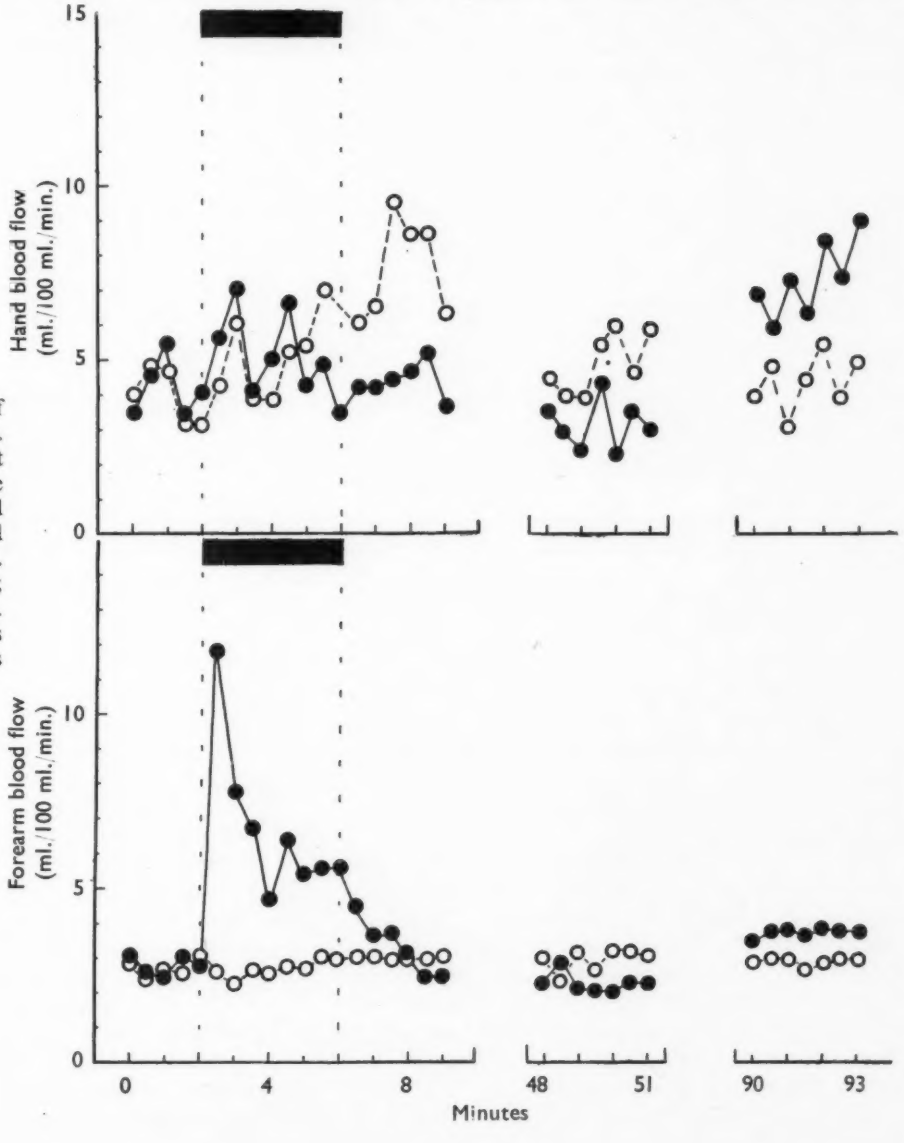

FIG. 1.—The effect of infusion of 12.5 mg. bretylium into the left brachial artery on the blood flow in the hand (upper panel) and forearm (lower panel). ●—● Left side; o—o right side. The black rectangles represent the periods of infusion.

normally as possible. This causes reflex vasoconstriction in the hand (Bolton, Carmichael, and Stürup, 1936).

Postural Changes.—The subject's legs were passively raised from the horizontal and maintained in a nearly vertical position for 2 to 3 min. This causes inhibition of vasoconstrictor tone in skeletal muscle (Roddie and Shepherd, 1956; Roddie, Shepherd, and Whelan, 1957c).

In one experiment the radial, median and ulnar nerves were blocked above the elbow with 3% ethocaine hydrochloride containing 0.002% adrenaline as described by Roddie, Shepherd, and Whelan (1957d).

Results

Effect of Bretylium on Forearm and Hand Blood Flow

The effect of an infusion of 12.5 mg. of bretylium on forearm blood flow is shown in Fig. 1 (lower panel). During the infusion there was a large transient and a smaller sustained increase in flow in the forearm infused with bretylium but not the control forearm. In one experiment 12.5 mg. bretylium was infused into the brachial artery on four occasions at 15 min. intervals. A quantitatively similar increase was seen on each occasion. After the infusion the flow fell to just below the level in the control forearm. There was little change in flow over the next 40 min. but after 80 min. the flow on the infused side was just above the control level. The results of 11 experiments illustrating the pattern of blood flow response following an infusion of bretylium are summarized in Fig. 2. Though there was considerable variation from subject to subject there was usually a fall in flow for about 30 min. but during the next hr. the flow returned to or above the previous resting level.

The response of the hand blood vessels to a similar infusion is shown by the experiment illustrated in Fig. 1 (upper panel) and the results of 7 such experiments are summarized in Fig. 3. During and immediately after the infusion flow was either little changed or reduced. About 1 hr. later, however, the blood flow on the experimental side had risen considerably above that on the control side. The size and time of onset of this increase varied greatly from subject to subject.

In one experiment forearm blood flow was measured in both arms, and the median, radial and ulnar nerves to one forearm were blocked above the elbow. This caused a large increase in blood flow on the blocked side (Fig. 4). Bretylium (12.5 mg.) was infused into the nerve-blocked side. A vasodilatation was seen during the infusion, but the flow after the infusion was much lower than the pre-infusion level.

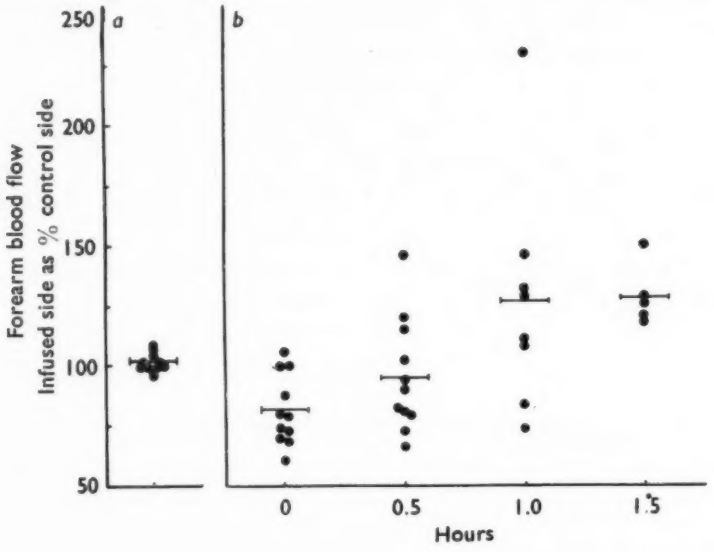

FIG. 2.—The effect of intra-arterial infusion of 12.5 mg. bretylium between *a* and *b* on blood flow in the forearm in 11 subjects. The blood flow on the infused side is expressed as a percentage of that in the control forearm. The dots represent the average values obtained over the 5 min. periods immediately before and after bretylium infusion and at subsequent 30 min. intervals.

Thirty min. after a second infusion the flows on the two sides were approximately equal, yet at this time muscular paralysis and skin anaesthesia were still complete.

Effect of Bretylium on Vascular Reflexes in the Forearm

Fig. 5 illustrates the effect of an intra-arterial infusion of 12.5 mg. of bretylium on some forearm vascular reflexes. After the infusion (1) the subject performed the Valsalva manœuvre (2). The usual vasoconstriction was seen on the control side, but there was a vasodilatation on the experimental side. During the period (3) emotional stress was produced by mental arithmetic; this caused an increase in flow in both the control and infused sides. The flows

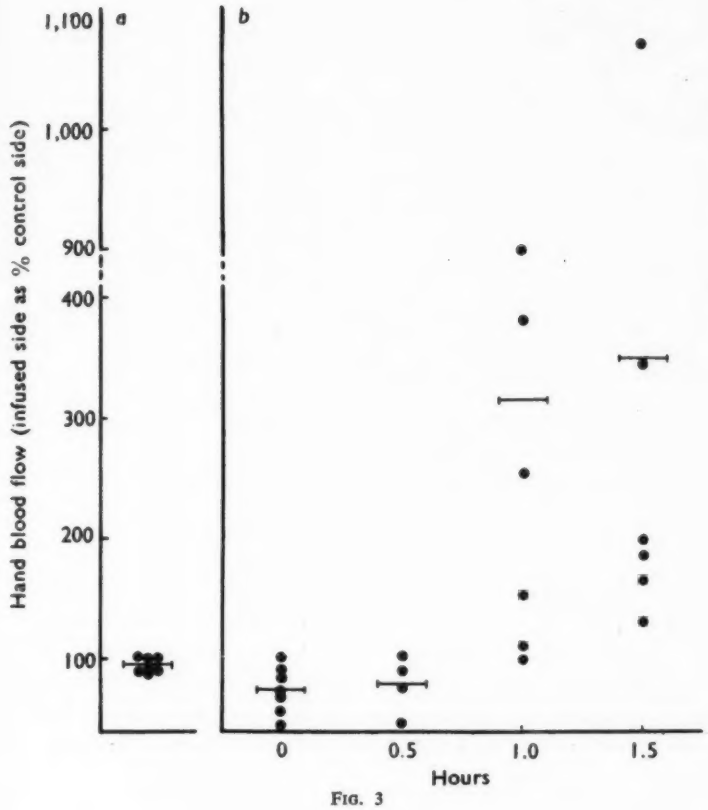

FIG. 3

FIG. 3.—The effect of intra-arterial infusion of 12.5 mg. bretylium between *a* and *b* on hand blood flow in 7 subjects.

FIG. 4.—The effect of intra-arterial infusion of bretylium on blood flow in the nerve-blocked forearm. At ↑ the radial, ulnar and median nerves to the left forearm were blocked. The black rectangles represent the periods when 12.5 mg. bretylium was infused into the left brachial artery. ●——● Left forearm blood flow; O——O right forearm blood flow.

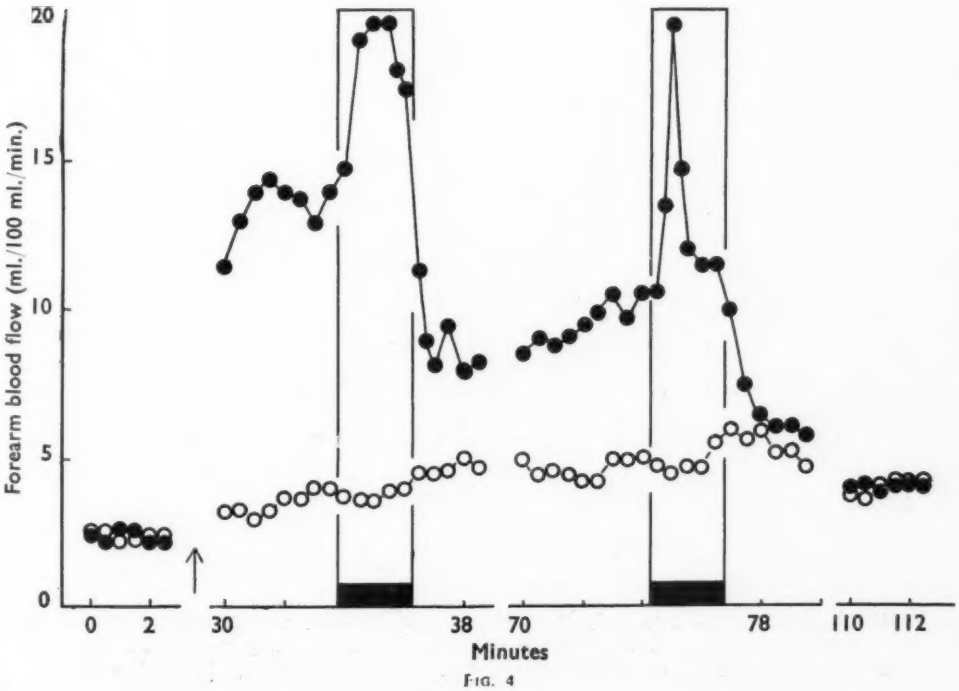

FIG. 4

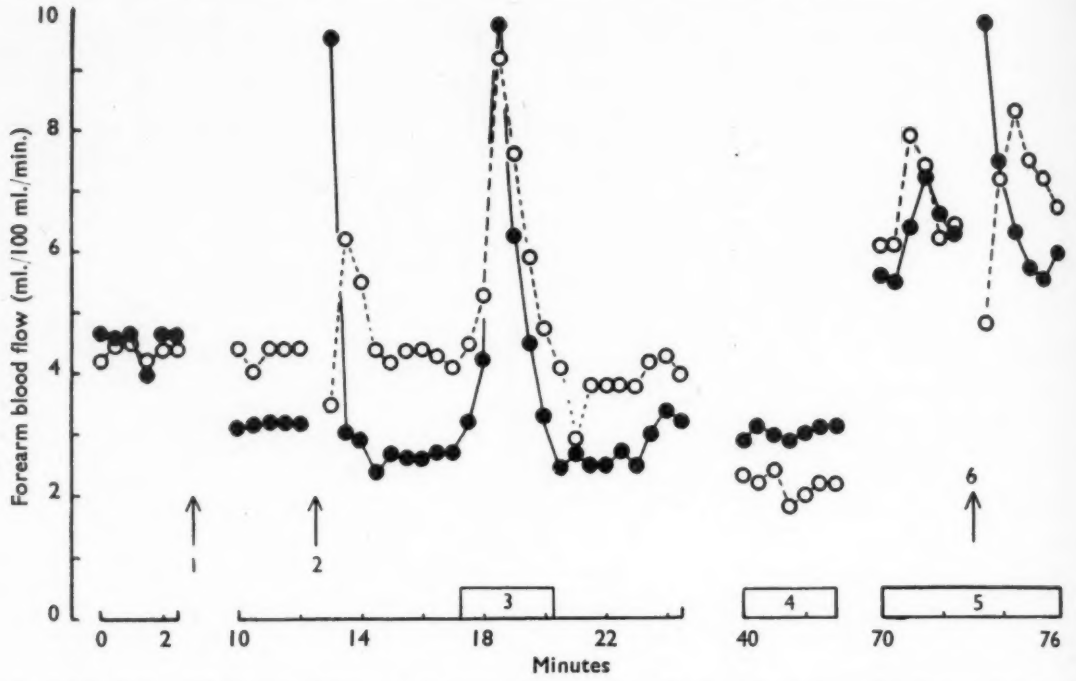

FIG. 5.—The effect of infusion of 12.5 mg. bretylium at (1) into the left brachial artery on the changes in blood flow in the forearm following the Valsalva manœuvre (2, 6) and during a mental arithmetic test (3), body cooling (4) and body heating (5). ●——● Left forearm blood flow; O——O right forearm blood flow.

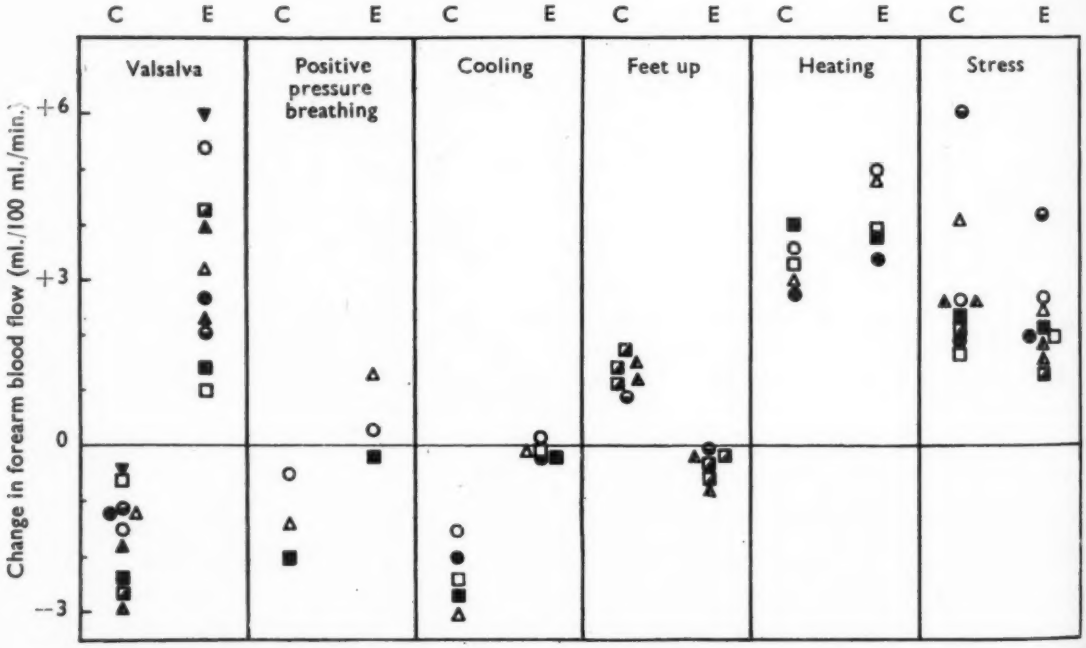

FIG. 6.—The effect of infusion of 12.5 mg. bretylium into the brachial artery on the changes in forearm blood flow following the Valsalva manœuvre and during positive pressure breathing, body cooling, body heating, emotional stress and passive raising of the legs of a recumbent subject. C, control forearm. E, experimental forearm.

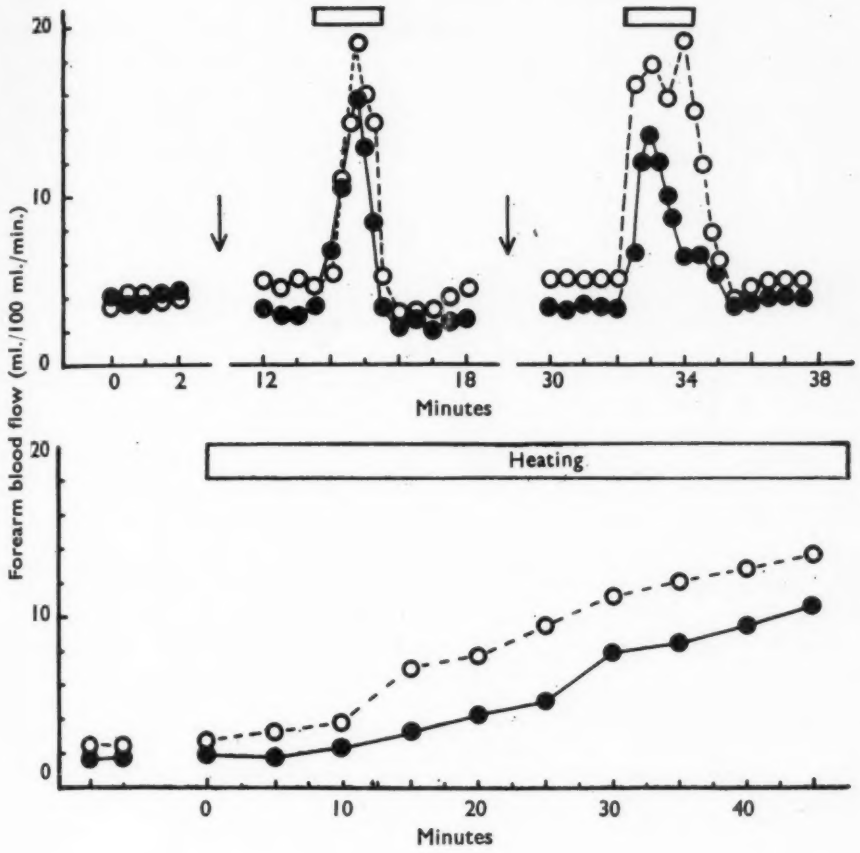

FIG. 7.—The effect of large doses of bretylium on the reflex responses in the forearm to emotional stress and body heating. Upper panel: 12.5 mg. bretylium was infused into the left brachial artery at first arrow and 37.5 mg. at the second arrow. During the periods represented by the open rectangles the subject was given a mental arithmetic test. Lower panel: 50 mg. bretylium was infused into the left brachial artery at the beginning of the experiment. The open rectangle represents the period when the body heating was carried out. ●——● Left forearm blood flow; O——O right forearm blood flow.

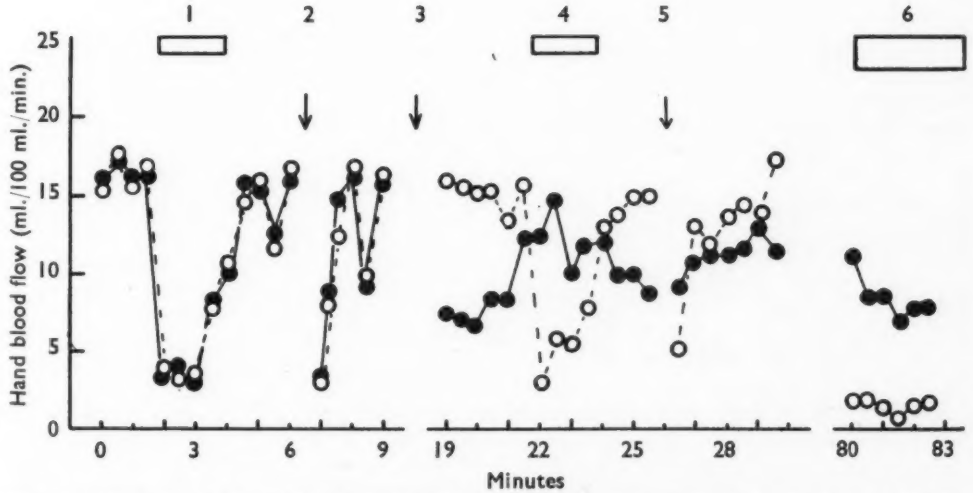

FIG. 8.—The effect of bretylium infusion (12.5 mg.) on vascular reflexes in the hand. During (1) the subject was given a mental arithmetic test. At (2) he made a deep inspiration and 12.5 mg. bretylium was infused into the left brachial artery at (3). At (4) and (5) the subject was given a mental arithmetic test and made a deep inspiration respectively. The flows during (6) were measured at the end of a period of body cooling.

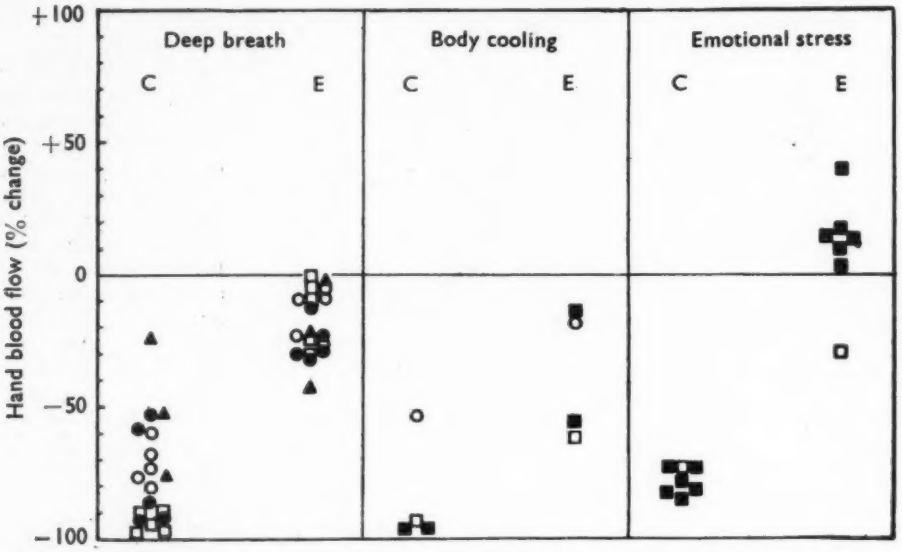

FIG. 9.—The effect of intra-arterial infusion of 12.5 mg. bretylium on the changes in hand blood flow during body cooling, emotional stress and following a deep breath. The mean blood flows during the tests in the control (C) and experimental (E) hands are expressed as percentages of the flow during the previous resting periods.

at (4) were measured after 15 min. of general body cooling. This caused a fall in flow on the control side but not the infused side. The flows at (5) were measured after a period of general body heating; there was an increase in flow in both forearms. The Valsalva manœuvre was repeated during this time (6), and the vasoconstrictor response was still abolished on the infused side. The results of all the experiments of this type on 10 subjects are summarized in Fig. 6. The vasoconstrictor response to the Valsalva manœuvre was reversed, and the responses to positive pressure breathing, leg raising and general body cooling were abolished on the infused side. However, this dosage of bretylium had little

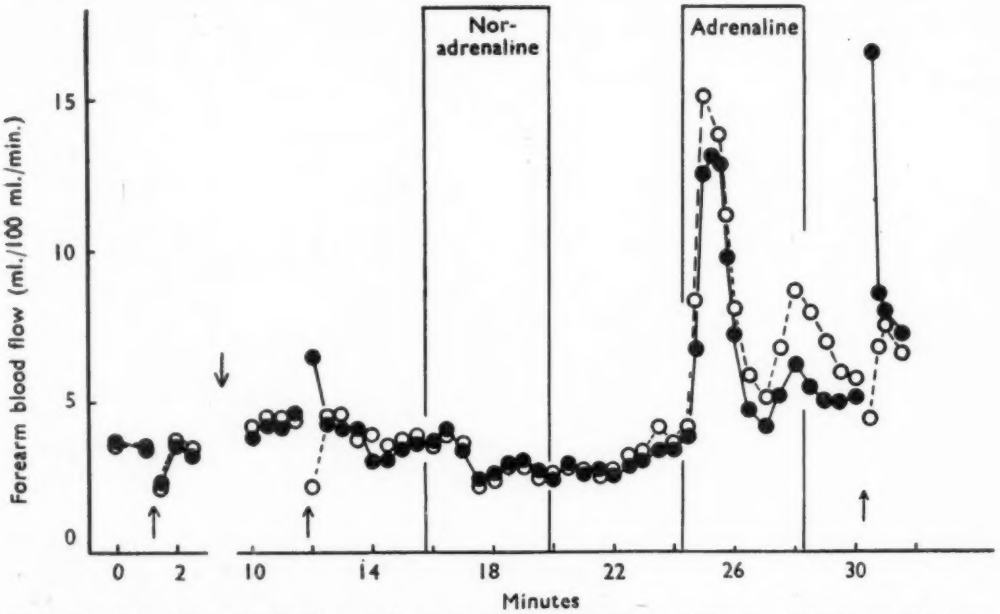

FIG. 10.—The effect of bretylium on the response of forearm blood flow to intravenous infusion of noradrenaline and adrenaline. Bretylium (50 mg.) was infused into the left brachial artery at the time indicated by the descending arrow. Valsalva manœuvres were performed at the times indicated by the ascending arrows. Adrenaline and noradrenaline (10 μg./min.) were infused during the periods represented between the vertical lines. ●——● Left forearm; O——O right forearm.

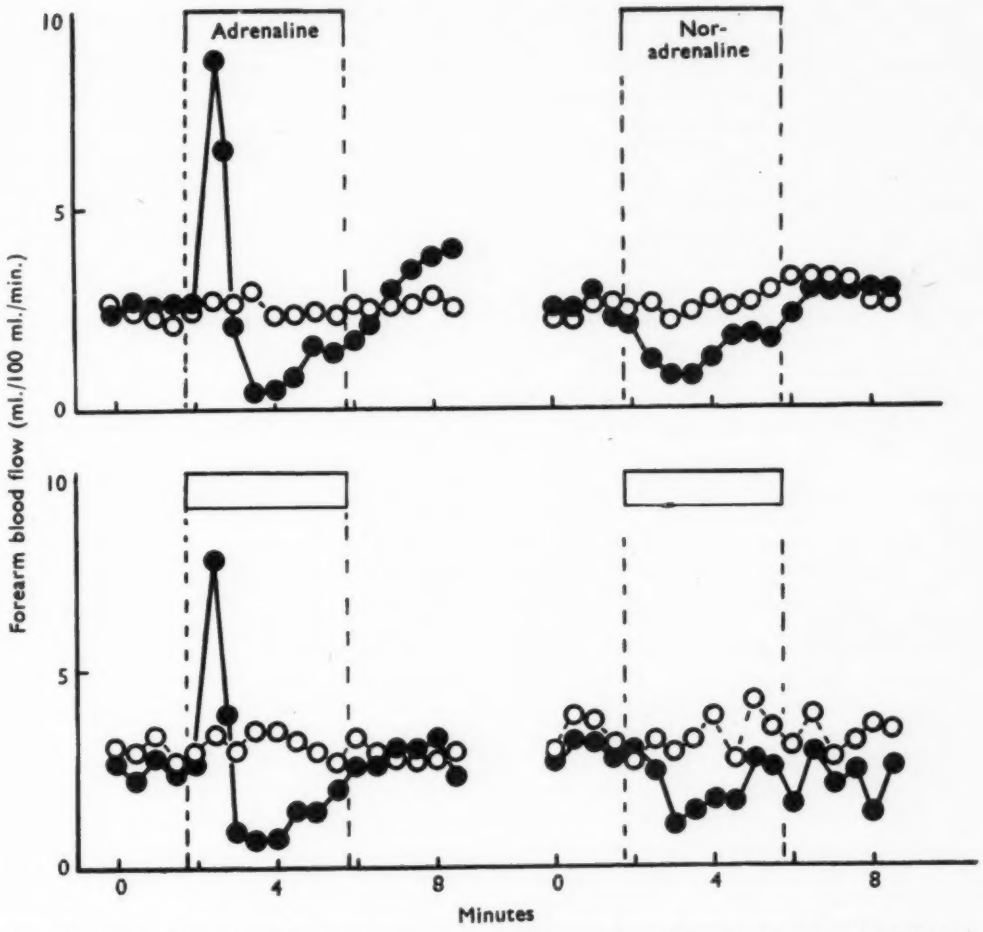

FIG. 11.—The effect of bretylium on the response of forearm blood flow to intra-arterial infusion of adrenaline and noradrenaline. Upper panel: before bretylium infusion. Lower panel: after bretylium infusion. Adrenaline and noradrenaline (1 μg./min.) were infused into the left brachial artery during the periods represented between the dotted vertical lines. ●——● Left forearm; O——O right forearm.

effect on the increase in forearm blood flow during emotional stress or general body heating.

Some experiments were performed to study the effect of large doses of bretylium on vasodilator reflexes in the forearm. In the experiment illustrated in Fig. 7a, 12.5 mg. of bretylium was infused into one forearm. This had no effect on the response to a mental arithmetic test. A further 37.5 mg. was infused into the same side, and the response to a mental arithmetic test was then reduced on the infused side. The same result was obtained in each of 2 similar experiments. In the experiment illustrated in Fig. 7b, 50 mg. bretylium was infused into one forearm, and general body heating was started at time 0. The vasodilatation was both delayed and reduced on the infused side. A similar result was obtained in one other experiment of this type. In neither case was sweating abolished on the infused side.

Effect of Bretylium on Vascular Reflexes in the Hands

Fig. 8 illustrates a typical experiment, and Fig. 9 summarizes the results of all of the experiments of this type on 5 subjects. The vasoconstrictor responses in the hands to mental arithmetic, a single deep breath and general body cooling were reduced or abolished following an intra-arterial infusion of 12.5 mg. of bretylium.

Effect of Bretylium on Response to Adrenaline and Noradrenaline

In the experiment illustrated in Fig. 10, 50 mg. bretylium was infused into the left brachial artery.

The formerly symmetrical Valsalva response was now reversed on the infused side, indicating successful sympathetic vasoconstrictor block. However, the response to the intravenous infusion of 10 μg./min. of noradrenaline and adrenaline respectively for 4 min. was similar on the two sides. Fig. 11 (upper panel) illustrates the response to an intra-arterial infusion of 1 μg./min. of adrenaline and noradrenaline. Bretylium (50 mg.) was then infused into the same side. The lower panel shows that the responses to subsequent adrenaline and noradrenaline infusions were unaffected by the bretylium. In 2 other experiments the response to noradrenaline only was tested in the same way. In one the vasoconstrictor response to noradrenaline was unchanged and in the other was slightly increased after bretylium.

Discussion

It is clear from the present experiments that the intra-arterial infusion of bretylium interferes with the reflex activity of vasoconstrictor nerves in the arm. Thus after infusion of bretylium there was no fall in forearm blood flow during body cooling or positive pressure breathing, or following the Valsalva manœuvre. Forearm blood flow did not increase when the subject's legs were passively raised. In the hand the vasoconstrictor responses to body cooling, mental arithmetic and a deep breath were either reduced or abolished. With such evidence of block of sympathetic vasoconstrictor nerves it was surprising that the blood flow often fell slightly following infusion of bretylium, for removal of sympathetic vasoconstrictor tone in the forearm (for example by acute nerve-block) is well known to increase the forearm blood flow 2 to 3 fold (Barcroft, Bonnar, Edholm and Effron, 1943). This suggested that bretylium had some action in addition to its blocking effect on sympathetic nerves. To study the direct effect of the drug, bretylium was infused into an acutely nerve-blocked forearm. Due to release of vasoconstrictor tone the blood flow through the nerve-blocked forearm was initially much higher than on the control side, but after the infusions of bretylium the flow fell to that in the control forearm, although at this time the nerve block was complete. This experiment indicated that in the absence of vasoconstrictor tone bretylium causes considerable vasoconstriction. This sympathomimetic effect could be due to the direct action of bretylium on the blood vessels or on the sympathetic nerve endings. The sharp initial vasodilatation and the smaller sustained vasodilatation seen during the infusion of bretylium also occurred in the nerve-blocked forearm, so these changes must be due to the direct action of the drug and not to release of vasoconstrictor tone.

The changes in flow after an infusion of bretylium were qualitatively similar in the hand and forearm. The flow was at first below that on the control side, but after 30 to 60 min. rose slightly above it. The state of the vessels at any time following infusion seemed to depend on the balance between the sympathomimetic action of the drug, and its sympathetic blocking effect. Immediately after infusion the sympathomimetic effect predominated but appeared to wear off over the next hour or so. This would explain why there is often an initial pressor response to bretylium (Boura and Green, 1959).

It is clear that the sympathomimetic effect is initially strong enough to compensate for the removal of the sympathetic tone present when in the supine position, since vasodilatation does not occur. The finding, that there is no great fall in peripheral resistance in the limbs, is in keeping with the observation that bretylium causes little fall in supine blood pressure (Boura *et al.*, 1959). However, because of the sympathetic blocking action, the usual increase in vasoconstrictor tone on adopting the erect posture is not possible; thus postural hypotension results.

In the animal experiments (Boura and Green, 1959) it was found that bretylium did not interfere with transmission at autonomic ganglia or at most caused only transient cholinergic block. It has been shown that the increase in blood flow that occurs in forearm skin during general body heating can be delayed and reduced by atropine (Roddie *et al.*, 1957a), and is therefore due, at least in part, to a cholinergic mechanism. With small doses of bretylium, the response to general body heating was little affected. After larger doses there was some reduction in the vasodilatation during body heating, but sweating was not obviously reduced. This suggests that cholinergic block, if it occurs at all, is very slight.

The vasodilatation in human skeletal muscle during emotional stress has lately been attributed in part to activation of cholinergic fibres (Blair, Glover, Greenfield and Roddie, 1959). In the present experiments the vasodilator response to a mental arithmetic test was often found to be unaffected at a time when other tests showed vasoconstrictor block to be present. These experiments provide mutual support for the findings that bretylium has little effect on cholinergic nerves, and also that vasodilatation can occur in the forearm during stress without involving the release of vasoconstrictor tone. In some experiments, particularly

with large doses of bretylium, there was some reduction in the response to stress on the infused side. This could be due to a slight degree of cholinergic block. Alternatively, reflex release of vasoconstrictor tone may occasionally contribute to the changes seen in the forearm during stress.

The present experiments indicate that the mode of action of bretylium is similar in man to that described in animals. Doses of bretylium which caused vasoconstrictor block did not reduce the responses to adrenaline or noradrenaline, given either intravenously or intra-arterially, and in one case the vasoconstrictor response to noradrenaline was increased. These results therefore provide further evidence that the action of the drug is sympatholytic rather than adrenolytic.

We wish to thank Dr. D. A. Long, of Burroughs Wellcome, for kindly supplying us with bretylium tosylate.

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THE ANTAGONISM OF MUSCLE RELAXANTS BY AMBENONIUM AND METHOXYAMBENONIUM IN THE CAT

BY

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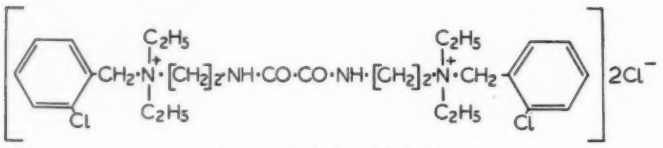
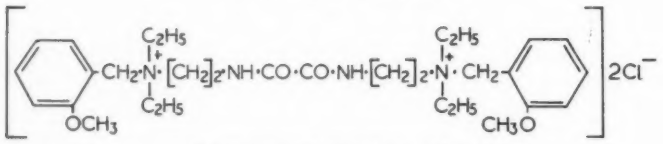
The ability of ambenonium and a methoxy analogue to antagonize paralysis produced either by tubocurarine or by decamethonium has been studied in the tibialis anterior muscle of the cat under chloralose anaesthesia. In small doses, both oxamides facilitated neuromuscular transmission, but in larger doses they depressed the sensitivity of the motor end plates to depolarizing substances and it is considered that this latter action is sufficient to account for their anti-decamethonium action. Although both compounds possess anticholinesterase activity, there was found to be no correlation between their relative abilities to antagonize tubocurarine paralysis and their abilities to inhibit muscle cholinesterase *in vitro*.

Ambenonium chloride (Mytelase; WIN 8077) and its methoxy analogue (WIN 8078) are members of a series of bisquaternary oxamides, synthesized in the Sterling-Winthrop Laboratories and first reported by Arnold, Soria, and Kirchner (1954).



Ambenonium chloride



Methoxyambenonium chloride

Both compounds have been shown to produce marked effects upon neuromuscular transmission (Arnold *et al.*, 1954; Lands and Karczmar, 1955; Lands, Karczmar, Howard, and Arnold, 1955; Lands, Hoppe, Arnold, and Karczmar, 1957; Karczmar, 1957; Lands, Hoppe, Arnold, and Kirchner, 1958) and were considered worthy of further study in the present experiments, particularly since one of them, methoxyambenonium, has been shown to be capable of antagonizing neuromuscular block produced in the tibialis anterior muscle of the cat both by tubocurarine and by decamethonium (Karczmar, 1957).

Methods

The experiments were carried out with 48 adult cats (1.6 to 4.9 kg.) anaesthetized with intravenous chloralose (80 mg./kg.).

For experiments on innervated muscles, shielded silver electrodes were placed on the sciatic nerve of one hind-limb and the nerve was ligated centrally to the electrodes. The limb was set up in a horizontal position on a Brown-Schuster myograph stand, and the tendon of the tibialis anterior muscle, and for certain experiments that of the soleus, were attached to flat steel spring myographs. Twitches, which were recorded on smoked paper, were excited by rectangular pulses of 0.2 msec. duration and of twice the strength required to evoke a maximal twitch. In a few experiments action potentials were simultaneously recorded from the tibialis anterior muscle by means of glass mounted platinum electrodes inserted through belly and tendon. After differential amplification by a Tektronix (type 122) battery-driven pre-amplifier, the action potentials were displayed on a Tektronix (type 502) double beam oscilloscope and photographed on 35 mm. film. For experiments on denervated muscle, the sciatic nerve was divided under pentobarbitone sodium anaesthesia and degeneration was allowed to proceed for from 17 to 19 days.

The drugs used were tubocurarine chloride, acetylcholine chloride, ambenonium chloride, methoxyambenonium chloride, decamethonium iodide and physostigmine salicylate. The doses quoted in the text refer to the salts. All drugs were dissolved in 0.15 M sodium chloride solution and were injected intravenously or intra-arterially. Since the main drugs under study

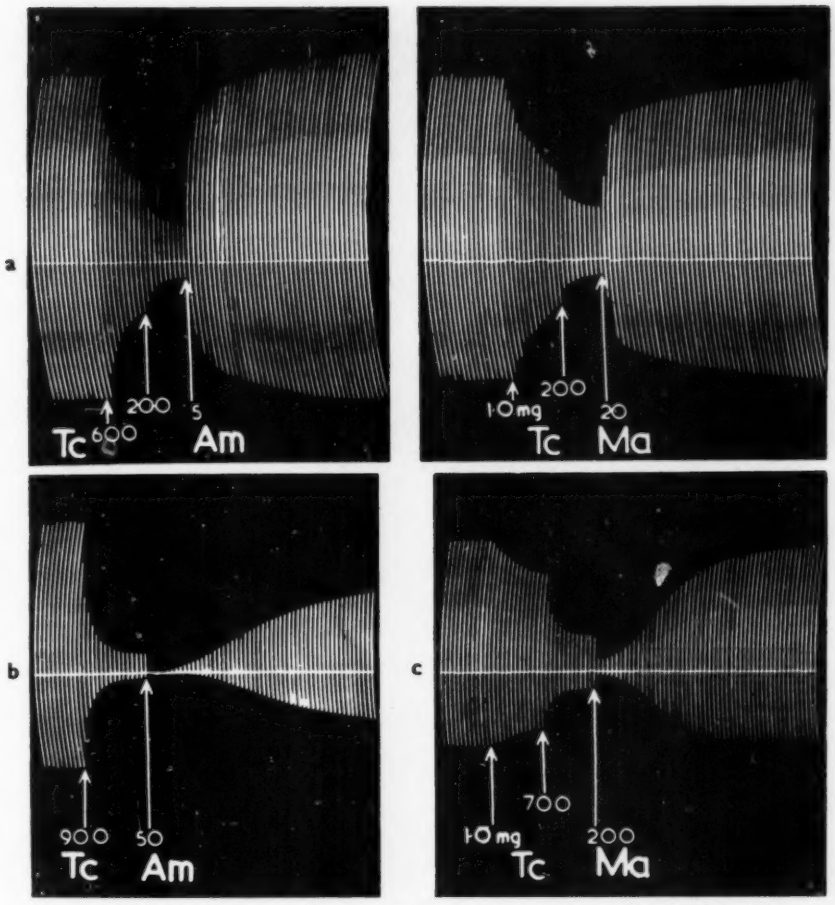


FIG. 1.—(*a*) Cat 1.75 kg.; (*b*) cat 3.3 kg.; (*c*) cat 4.9 kg. Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. At Tc, tubocurarine was injected intravenously. At Am, ambenonium, and at Ma, methoxyambenonium was injected intra-arterially. All doses in μg. unless otherwise stated.

were always injected intra-arterially close to the muscle, no adjustment for body weight was made. Intra-arterial injections were made towards the heart, in a volume of 0.2 ml. into the tibial artery below the tibialis anterior muscle, and at the moment of injection the femoral artery was occluded just peripheral to the saphenous branch; the injected drug therefore reached both the tibialis anterior and the soleus muscle. At the beginning of all experiments atropine sulphate (1.5 mg./kg.) was administered intraperitoneally, and arterial blood pressure was always recorded throughout experiments.

Cholinesterase was estimated manometrically using the Warburg apparatus. Cat tibialis anterior muscles were homogenized in an all-glass homogenizer to give a concentration of 150 mg./ml. in 0.04 M sodium bicarbonate (adjusted to *p*H 7.6 by the addition of hydrochloric acid) and 2.0 ml. of this homogenate were used for each estimation. The inhibitors were dissolved in 0.04 M sodium bicarbonate. Acetylcholine chloride was used as the substrate, the final concentration being 0.0138 M (Aldridge, 1950). The total volume of fluid in each flask was 3.0 ml. The flasks were gassed with a mixture containing 95% N_2 and 5% CO_2 for 10 min. and the manometers were read at 10 min. intervals for 30 min.

RESULTS

Effects on Tubocurarine Paralysis

In 6 experiments tubocurarine was administered intravenously in doses sufficient to reduce the indirectly excited maximal twitches of the tibialis anterior muscle by approximately 80% and ambenonium or methoxyambenonium was administered intra-arterially at the time of maximum block. The smallest dose of ambenonium necessary to produce a distinct anti-curare effect was of the order of

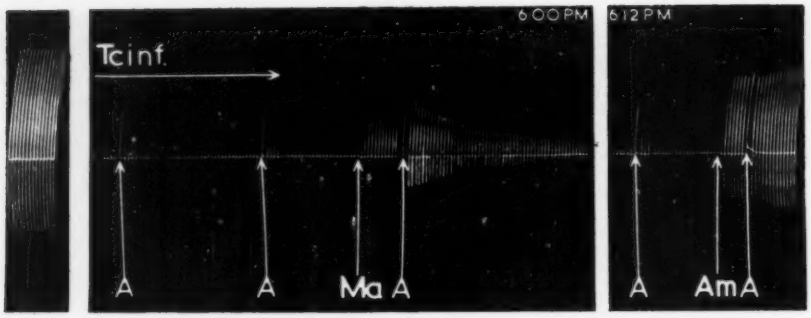


FIG. 2.—Cat 4.0 kg. Maximal twitches of the tibialis anterior muscle elicited indirectly once every 10 sec. A constant partial paralysis was maintained by a continuous intravenous infusion of tubocurarine (1.8 mg./hr.). At A, 100 μg. acetylcholine; at Ma, 10 μg. methoxyambenonium; and at Am, 3 μg. ambenonium injected intra-arterially.

0.05 μg. and the maximal effect was obtained with doses of 4 to 5 μg. Methoxyambenonium also antagonized tubocurarine paralysis, the corresponding doses being 0.2 μg. and 10 to 20 μg. respectively.

With further increase in the dosage of both compounds the antagonistic effect became progressively smaller until, with doses of the order of 50 μg. of ambenonium and 200 μg. of methoxyambenonium, the effect was reversed, both compounds then further deepening the paralysis produced by tubocurarine. Fig. 1 illustrates some of these results.

In 4 other experiments, a constant partial paralysis was maintained by a continuous intravenous infusion of tubocurarine. At intervals throughout the experiments, electrical stimulation was temporarily stopped and acetylcholine in a dose sufficient to produce a submaximal contraction was injected close-arterially. Ambenonium (3 to 5 μg.) or methoxyambenonium (10 to 20 μg.) was then injected and their effects on twitch tension and on the contraction produced by acetylcholine were studied. Both compounds caused a marked increase in the tension of the partially blocked twitches, but only ambenonium potentiated the contraction produced by injected acetylcholine. Fig. 2 illustrates these results.

Effects on Decamethonium Paralysis

Decamethonium blocks the tibialis anterior muscle of the cat by long lasting depolarization of the motor end plates (Zaimis, 1951; Burns and Paton, 1951). In the soleus muscle, on the other hand, the drug produces what Jewell and Zaimis (1954) called a dual mode of action block. That is, the substance starts its action by depolarizing the motor end plates, but during the blocking process the action changes and the block shows many of the characteristics of that produced by tubocurarine. For this reason, in 5 of these 15 experiments, maximal twitches of both muscles were recorded simultaneously. Decamethonium was administered intravenously in doses sufficient to reduce the twitch tension of the tibialis anterior muscle by approximately 80% and ambenonium and methoxyambenonium were injected intra-arterially at the height of the paralysis.

The effects produced by the oxamides in the soleus muscle closely resembled those already described for the tibialis anterior muscle when tubocurarine was the blocking agent used. Thus, small doses (5 μg. of ambenonium and 20 μg. of methoxyambenonium) antagonized the paralysis while larger doses (100 μg.) caused a further deepening. In the tibialis anterior muscle, small doses of ambenonium (0.2 to 1 μg.) often caused a small increase in the tension of the partially blocked twitches. With larger doses (5 to 10 μg.) the effect was reversed and a small decrease in tension was produced. Further increase in the dosage (20 to 50 μg.) again reversed the effect, with the result that a striking antagonism of the block occurred. With very large doses (200 μg. and above) the paralysis was again deepened, but later experiments showed that this effect was due to conversion of the decamethonium block to that of ambenonium itself.

Minimal effective doses of methoxyambenonium (4 to 5 μg.) antagonized the decamethonium block in the tibialis anterior muscle and this response increased with increase in dose, up to a maximum with doses in the range of 50 to 100 μg. Very large doses (400 μg. and above) caused a further deepening of the paralysis, but, as with ambenonium, this effect was shown to be due to a conversion from the

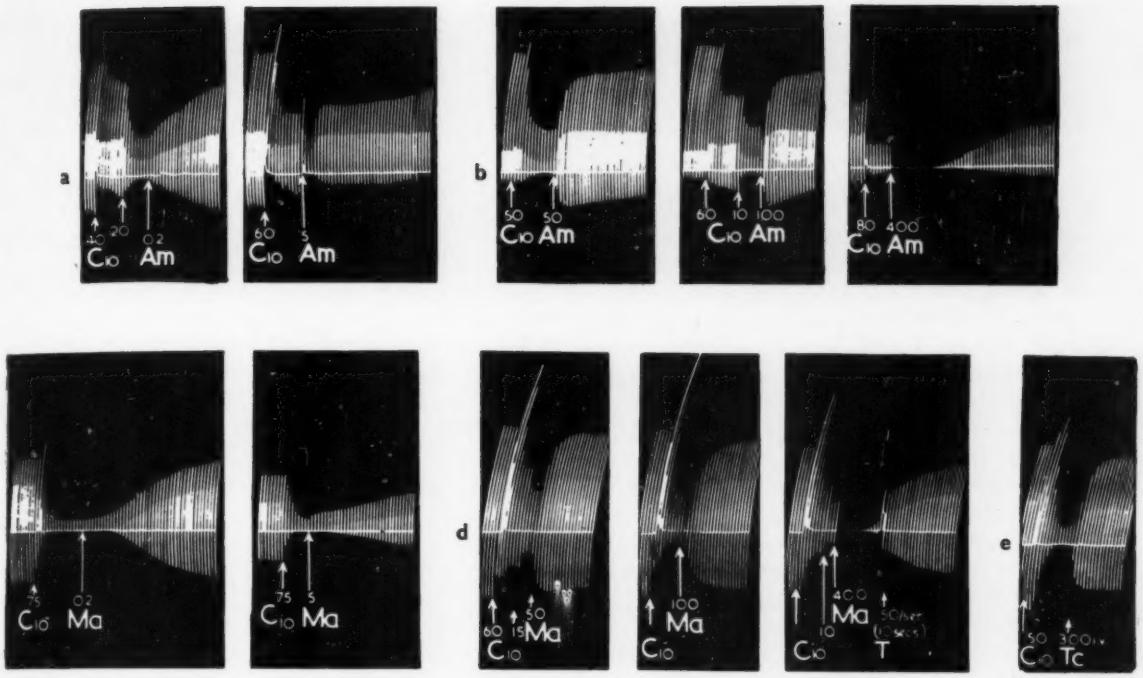


FIG. 3.—(*a*), Cat 2.8 kg.; (*b*) cat 3.1 kg.; (*c*) cat 3.1 kg.; (*d*) cat 2.3 kg.; (*e*) cat 3.0 kg. Maximal twitches of the tibialis anterior muscles elicited indirectly once every 10 sec. All doses in μg. At C_{10}, decamethonium was injected intravenously. At Am, ambenonium, and at Ma, methoxyambenonium were injected intra-arterially. At Tc, tubocurarine intravenously, and at T, tetanus 50/sec. for 10 sec.

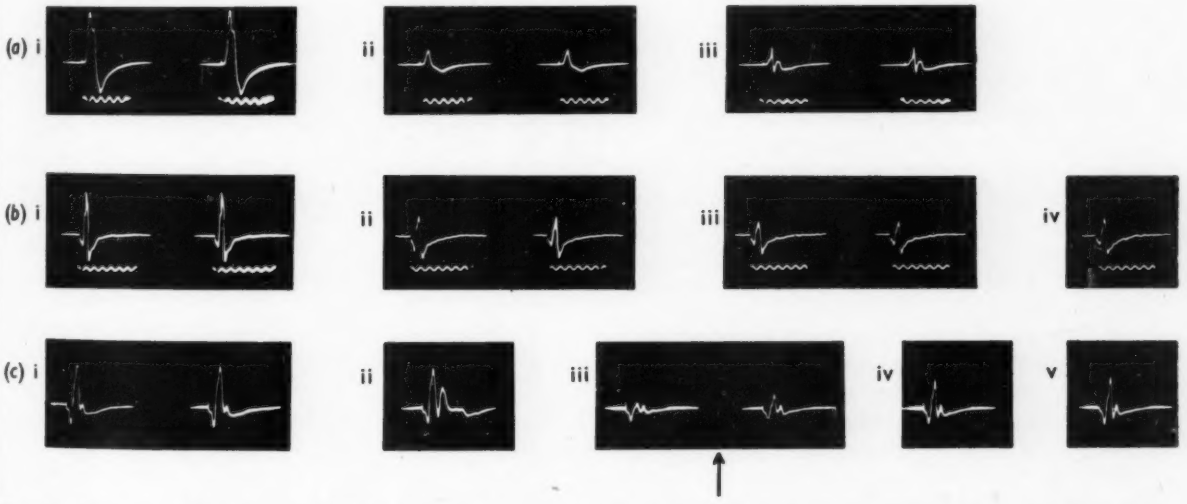


FIG. 4.—Maximal twitches of the tibialis anterior muscles were elicited indirectly once every 10 sec. (*a*) Cat 3.1 kg.; (i) normal muscle action potentials; (ii) action potentials recorded at the peak of the paralysis produced by the intravenous injection of 60 μg. decamethonium; and (iii) repetitive action potentials recorded 30 and 40 sec. after the intra-arterial injection of 1.0 μg. ambenonium. (*b*) Cat 5.0 kg.; (i) normal; (ii) at peak of paralysis produced by 75 μg. decamethonium; (iii) 20 and 30 sec. after intra-arterial injection of 10 μg. ambenonium; (iv) 100 sec. later. (*c*) Cat 3.2 kg.; (i) normal; (ii) repetitive firing elicited by 75 μg. decamethonium; (iii) at peak of decamethonium paralysis; at the arrow (↑) 100 μg. methoxyambenonium injected intra-arterially; (iv) 30 sec. later; (v) 100 sec. later. Time trace, 200 c/s.

decamethonium block to that of methoxyambenonium itself. These effects on the tibialis anterior muscle are illustrated in Fig. 3.

Recording of the gross action potential in tibialis anterior showed that repetitive firing initiated by decamethonium occasionally persisted in some muscle fibres throughout a partial neuromuscular block. The small increase in the twitch tension of the partially blocked muscle caused by very small doses of ambenonium (0.2 to 1 μg.) was accompanied by increased repetitive firing in some of those fibres which remained unblocked. The small increase in tension was not a result of more muscle fibres contributing to the gross tension, because the peak voltage of the action potential was not increased at this dose level. Larger doses of ambenonium (5 to 10 μg.), which deepened the paralysis produced by decamethonium, decreased the peak voltage of the action potential. Still larger doses (20 to 50 μg.) produced a true antagonism as shown by the fact that the peak voltage of the action potential was increased. With methoxyambenonium, the first two responses were absent and doses from 5 to 100 μg. increased the peak voltage of the gross action potential. Methoxyambenonium was therefore a more potent antagonist of decamethonium paralysis than ambenonium. These changes in the gross action potential are illustrated in Fig. 4.

Effects on Maximal Twitch and on Contractions Produced by Acetylcholine

In 9 experiments doses of ambenonium (3 to 5 μg.) previously shown to antagonize tubocurarine always potentiated both the indirectly excited maximal twitches and the contractions produced by intra-arterially injected acetylcholine (Fig. 5*a*). With larger doses (100 μg. and above), the maximal twitch tension was depressed and the responses to acetylcholine were abolished (Fig. 6*a*). In contrast, methoxyambenonium did not potentiate either response in any dose. The smallest effective dose (10 μg.) caused a depression of the contractions produced by acetylcholine (Fig. 5*b*). Large doses (200 μg. and above) depressed the maximal twitch tension and completely abolished the response to injected acetylcholine (Fig. 6*b*). During the depression of the indirectly excited maximal twitches produced either by ambenonium or by methoxyambenonium, direct stimulation of the muscle produced a normal contraction. Even in the maximum dose administered (800 μg.), neither of the oxamides by themselves caused a contraction of the muscle.

In 12 experiments on chronically denervated muscles, the only effect of the oxamides was to reduce the contracture produced by intra-arterially administered acetylcholine. Once again there was no evidence of a direct stimulant action of the compounds.

In a further series of 8 experiments, the characteristics of the block produced by large doses of the oxamides themselves were studied. Since ambenonium in particular is known to be a potent anticholinesterase (Lands *et al.*, 1955; Koelle, 1957) the blocking effects of both compounds were compared with that of physostigmine. During neuromuscular block produced by physostigmine, tetanic tension was not maintained and after the tetanus the paralysis was temporarily deepened. The administration of acetylcholine, decamethonium or tubocurarine also deepened the paralysis produced by physostigmine. In contrast, during partial paralysis produced by ambenonium or methoxyambenonium

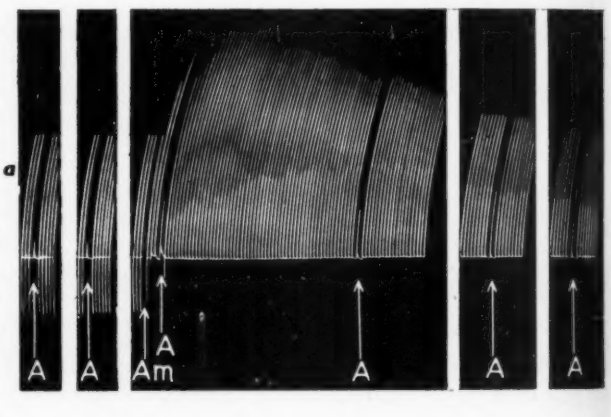


FIG. 5.—(*a*) Cat 3.2 kg.; (*b*) cat 2.6 kg. Maximal twitches of the tibialis anterior muscles elicited indirectly once every 10 sec. In (*a*) A=4 μg. and in (*b*) A=8 μg. acetylcholine. At Am, 3 μg. ambenonium, and at Ma, 10 μg. methoxyambenonium were administered. All injections intra-arterially.

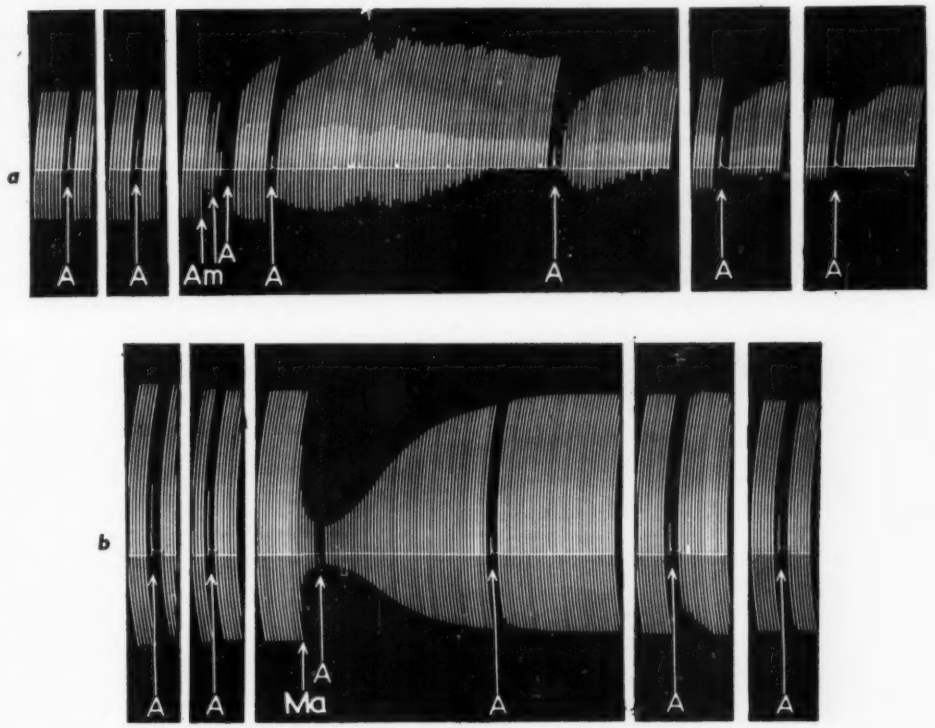


FIG. 6.—(*a*) Cat 2.15 kg.; (*b*) cat 3.6 kg. Maximal twitches of the tibialis anterior muscles were elicited every 10 sec. in (*a*), A=5 μg., and in (*b*), A=10 μg. acetylcholine. At Am, a total of 400 μg. ambenonium, and at Ma, 800 μg. methoxyambenonium was administered. All injections intra-arterially.

tetanic tension was well maintained and, after the tetanus, the paralysis was temporarily antagonized. Furthermore, although the block was deepened by the administration of tubocurarine, it was antagonized by the depolarizing substances, acetylcholine and decamethonium.

The experiments illustrated by Fig. 7 demonstrate the contrast between paralysis produced by physostigmine and that produced by methoxyambenonium.

Owing to the potent anticholinesterase action of ambenonium, antagonism of its blocking action was seen only with small doses of acetylcholine (approximately 10 μg.). With larger doses, the block was converted to that of acetylcholine itself. In contrast larger doses of acetylcholine (100 μg. and above) were required to antagonize the paralysis produced by methoxyambenonium. Such doses are similar to those necessary to antagonize tubocurarine paralysis (Hutter, 1952; Blaber and Bowman, 1959). The block produced by the oxamides was not antagonized either by edrophonium or by neostigmine.

Anticholinesterase Activity

The *in vitro* activity of the oxamides was estimated, using tibialis anterior muscle homogenates as the source of enzyme. Koelle (1957) has shown that ambenonium and methoxyambenonium are reversible inhibitors of cholinesterase and the enzyme activity was therefore estimated in the presence of the inhibitors. The results obtained are expressed in Fig. 8 and show that throughout the whole range of concentrations ambenonium was approximately 100 times more potent than methoxyambenonium in its ability to inhibit the enzyme. The pI 50 values were 8.2 for ambenonium and 6.0 for methoxyambenonium. These figures closely agree with those reported by Koelle (1957), who used cat brain as the source of the enzyme.

DISCUSSION

The results obtained confirm Karczmar's (1957) finding that methoxyambenonium is capable of antagonizing neuromuscular block produced in the tibialis anterior muscle of the cat either by tubo-

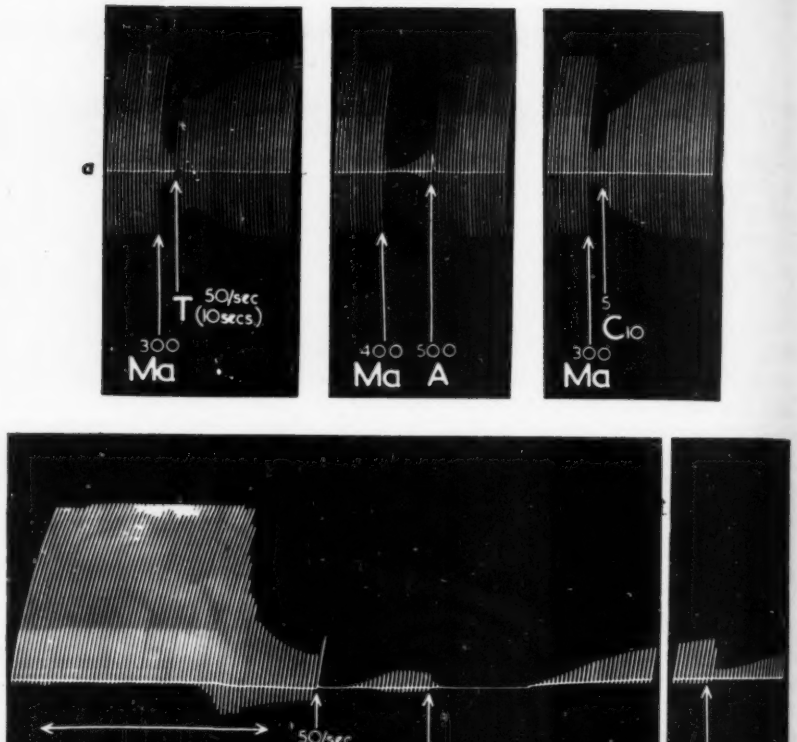


FIG. 7.—(*a*) Cat 4.9 kg.; (*b*) cat 3.2 kg. Maximal twitches of the tibialis anterior muscles elicited indirectly once every 10 sec. At Ma, methoxyambenonium; at A, acetylcholine; at C_{10}, decamethonium; and at P, physostigmine were administered. All doses are in µg. intra-arterially unless otherwise stated. At T, a tetanus was elicited by stimulation of the motor nerve.

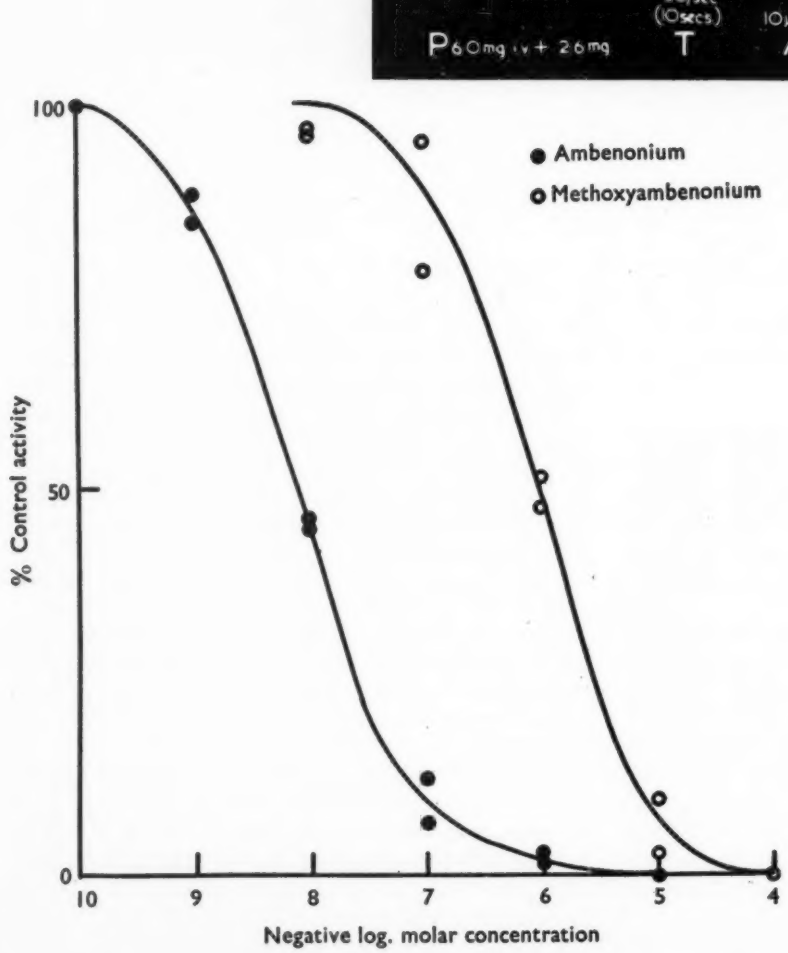


FIG. 8.—Inhibition of cholinesterase activity of cat tibialis anterior muscle homogenate by ambenonium and methoxyambenonium.

curarine or by decamethonium; in addition, they show that ambenonium also produces these effects. Tubocurarine and decamethonium are believed to cause paralysis in the tibialis anterior muscle of the cat by entirely different modes of action, but since the same compound could antagonize both, Karczmar concluded that his results cast serious doubt on the currently accepted theories of neuromuscular block produced by these drugs. However, as the present experiments emphasize, much larger doses than those required to antagonize tubocurarine are needed to produce a comparable relief of decamethonium paralysis, and at the higher dose level an entirely different action of the oxamides comes into play. Indeed the large doses capable of producing a marked antagonism to decamethonium paralysis actually deepened that produced by tubocurarine.

In the normal muscle, large doses of the oxamides themselves blocked both the response to injected acetylcholine and the indirectly elicited maximal twitches. Although ambenonium is a potent inhibitor of cholinesterase, an analysis of the neuromuscular block produced by the oxamides showed that it differed markedly from that produced by physostigmine. Rather, it resembled in many respects that produced by tubocurarine. Thus the block was antagonized by tetanus, by acetylcholine and by other depolarizing substances. Unlike that produced by tubocurarine, block produced by the oxamides was not antagonized by neostigmine or edrophonium and high frequency stimulation of the motor nerve during the paralysis produced a well sustained tetanus. These differences can probably be accounted for by the fact that the oxamides possess a facilitatory action on neuromuscular transmission, in addition to their blocking action at the motor end plates. Whatever the detailed mechanism of their blocking action, however, the important factor is that large doses of the oxamides raise the threshold of the motor end plates to the action of depolarizing substances. This effect is therefore sufficient to explain their antagonistic action to decamethonium paralysis since small doses of tubocurarine (see Fig. 3*e*) will produce the same effect (Dallemagne and Philippot, 1952). Although ambenonium was more potent than methoxyambenonium in other respects, it was less potent as an antagonist of decamethonium paralysis. This can probably be explained by the powerful anticholinesterase action of ambenonium, since persisting acetylcholine would tend to increase the decamethonium block and thereby mask its antagonistic action at the motor end plates to some extent. In fact, potentiation of decamethonium paralysis was seen with intermediate doses of ambenonium but not of methoxyambenonium.

Small doses of the oxamides produced a powerful antagonism to tubocurarine paralysis, but only ambenonium increased the contraction produced by injected acetylcholine and potentiated the indirectly elicited maximal twitch tension in the unblocked muscle. These differences between the two oxamides are again explainable by the finding that ambenonium is a much more powerful anticholinesterase than methoxyambenonium. Throughout the whole range of effective concentrations, methoxyambenonium was found to be about 100 times less powerful than ambenonium in its ability to inhibit muscle cholinesterase, but in spite of this, it was only 3 to 4 times less effective in its ability to antagonize tubocurarine. These results add some support to the suggestion (Bowman, 1958; Blaber and Bowman, 1959) that cholinesterase inhibition is not the main mechanism responsible for the anti-curare action of anticholinesterases but that such inhibition does play an important part in their ability to potentiate the maximal twitch tension of unblocked muscle.

Methoxyambenonium did not potentiate the response of the innervated muscle to injected acetylcholine and neither oxamide potentiated that of the chronically denervated muscle. These results make it unlikely that an increase in the sensitivity of the effector cell plays any part in the anti-curare effect. Results obtained with other anticholinesterases led Bowman (1958) and Blaber and Bowman (1959) to suggest that a pre-synaptic action, through which the amount of acetylcholine liberated by a nerve impulse is increased, might play a part in their anti-curare action and a similar mechanism would explain the results obtained with ambenonium and methoxyambenonium in the present experiments.

In conclusion, it can be said that, although most of Karczmar's results have been confirmed, they are capable of a different interpretation and can readily be explained on a basis of the current theories of neuromuscular block produced by tubocurarine and decamethonium. In small doses the oxamides facilitate neuromuscular transmission in some way, as shown by their antagonism of tubocurarine paralysis. In larger doses they depress the sensitivity of the motor end plates to depolarizing substances and this accounts for their ability to antagonize decamethonium. The oxamides are not alone in their ability to produce these responses; other facilitatory substances, which in large doses possess a weak curare-like action, may be capable of the same effects. For example, tetraethyl-

ammonium causes a pronounced antagonism to tubocurarine paralysis (Stovner, 1958; Koketsu, 1958; Blaber and Bowman, 1959), but very large doses themselves produce a curare-like block of the indirectly excited maximal twitches and at this dose level the substance deepens block produced by tubocurarine and antagonizes that produced by decamethonium (Blaber and Bowman unpublished).

I am indebted to Bayer Products and to Sterling-Winthrop Research Institute for their generous gifts of ambenonium and methoxyambenonium respectively. My thanks are also due to Dr. W. C. Bowman for his constant advice and encouragement throughout this work, and to Mr. D. Tulett for technical assistance.

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THE ANTITUBERCULAR PROPERTIES OF A SERIES OF THIOLS AND SULPHIDES

BY

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The antitubercular activity of a series of thiols, dithiolans, thiol esters, dimercaptopropyl esters, and episulphides has been examined *in vitro* and *in vivo* in mice infected with the $H_{37}Rv$ strain of *Mycobacterium tuberculosis*. Most of the thiol compounds were inactive, although dimercaprol (2,3-dimercaptopropanol; B.A.L.) and a few closely related compounds showed slight activity *in vivo*, the only exception being 2,3-dimercaptopropyl chloride which was very active. The dithiolans were inactive, but some of the thiol esters were moderately active, in particular 2,3-di(acetylthio)propyl acetate and 1,2,3-tri(acetylthio)propane. The majority of the dimercaptopropyl esters had significant activity, the most active compounds being 2, 3 - dimercaptopropyl benzoate, 1, 3 - dimercapto - 2 - propyl benzoate, 2, 3 - dimercaptopropyl *o*-chlorobenzoate, and 2,3-dimercaptopropyl *p*-chlorobenzoate. All the *S*-acyl derivatives of 3-mercaptopropylene sulphide had good antitubercular activity, some being more active than streptomycin. The most active compound of the series was 3-(2-furoylthio)propylene sulphide. The activity of the compounds is believed to be due to their conversion *in vivo* to 3-mercaptopropylene sulphide and not due to the formation of ethanethiol. Slight deviation from the basic structure abolishes antitubercular activity.

Anderson and Chin (1947) first drew attention to the role of thiol compounds in experimental tuberculosis. These workers found that 2,3-dimercaptopropanol (dimercaprol; B.A.L.) 100 μg./ml. inhibited the growth of *Mycobacterium tuberculosis* grown in Dubos medium. Del Pianto (1950) reported the activity of 2-mercaptobenzothiazole in combination with salts of the *S*-esters of thiosulphonic acid. He suggested that the antitubercular activity of sodium ethyl thiosulphate was due to the formation of ethylmercaptan (ethanethiol). Solotorovsky and his colleagues [Brown, Matzuk, Becker, Conbere, Constatin, Solotorovsky, Winsten, Ironson and Quastel (1954); Solotorovsky, Winsten, Ironson, Brown and Becker (1954); and Solotorovsky, Winsten, Ironson and Brown (1956)] examined a series of ethylthio compounds, in particular *S*-ethyl-L-cysteine, which was reported to be more active than *p*-aminosalicylic acid when administered in the diet. Other workers were unable to confirm these findings and further investigation revealed that the activity of *S*-ethyl-L-cysteine and other members of the series depended upon the formation of the volatile compound ethanethiol [Solotorovsky (1955) (1956); Solotorovsky, Ironson, Winsten (1956); Oginsky, Solotorovsky and Brown (1955) (1956)]. Kushner, Dalalian, Bach, Centola, Sanjurjo and Williams (1955) and Davies, Driver, Hoggarth, Martin, Paige, Rose and Wilson (1956) have investigated more recently the antitubercular activity of series of compounds related to ethanethiol. The latter authors considered that only compounds which were converted to ethanethiol were active.

We have investigated the antitubercular activity of dimercaprol and a number of related compounds and have found some active derivatives. The activity of the compounds does not appear to depend upon the formation of ethanethiol.

METHODS

The *organism* employed was the human strain of *Mycobacterium tuberculosis*, $H_{37}Rv$, reference National Collection of Type Cultures 7416.

Compounds to be tested for *in vitro* activity were treated according to their solubility. Aqueous solutions were used when possible, but compounds which were only sparingly soluble in water were dissolved in 1 ml. ethoxyethanol and diluted 1:20 with distilled water. This ether possesses some growth-inhibitory activity against *Mycobacterium tuberculosis* at a concentration of 1:20, but the amount of ethoxyethanol in the final test concentration was never allowed to exceed 1:200.

Serial concentrations of the dissolved compounds were made in Dubos medium. The tubes were inoculated with two drops of 1:10 concentration of a

suspension, standardized to Wellcome opacity tube 2, of a 10-day-old culture of *Mycobacterium tuberculosis* grown in Dubos, and incubated at 37°. Readings of growth were made at 7 days.

The activity is recorded as the lowest concentration of the compound (μg./ml.) inhibiting growth of the organism, that is, the minimal inhibitory concentration (M.I.C.).

In vivo *Tests.*—Male albino mice weighing 18 to 22 g. were infected by injecting intravenously 0.2 ml./20 g. of a suspension in saline of a 15-day-old culture of $H_{37}Rv$ strain of *Mycobacterium tuberculosis* grown in Proskauer and Beck medium. The suspension opacity was adjusted to a density equivalent to 4 mg. wet weight of organism/ml.

Compounds were administered daily in arachis oil (unless otherwise stated—see Tables) at doses of 1/2, 1/5, 1/10, and 1/20th of their approximate subcutaneous LD50 values to groups of mice, 10 to a group. The arachis oil solution acted as a depot from which the compounds were slowly absorbed. In each experiment one group received no treatment and acted

TABLE I
INACTIVE THIOLS

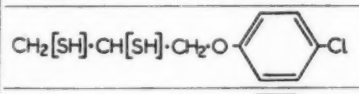
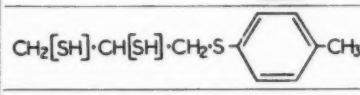
B.R.L. No.	Formula	B.R.L. No.	Formula
215	$CH_2(SH).CH_2.SH$	554	$[CH_2]_3(S.CH_2.CH(SH).CH_3)_2$
217	$CH_2(SH).CH_2.CH_2.SH$		
218	$CH_2(SH).CH_2.CH_2.CH_2.SH$	559	$CH_3.CH(SH).CH_2.S.[CH_2]_3.SH$
231	$CH_2(SH).CH(OH).CH_2.SH$	560	$CH_2(OH).CH_2.CH_2.S.CH_2.CH(SH).CH_3$
243	$CH_2(OH).CH_2.SH$	570	$CH_2(SH).CH_2(OH).CH_2.S.CH_2.CH(SH).CH_3$
244	$CH_2(SH).CH(SH).CH_3$	573	$CH_2(SH).CH_2.S.CH_2.CH_2SH$
248	$CH_2(SH).CH(SH).CH_2.SH$	576	$CH_2(SH).CH(OH).CH_2.CH_2SH$
259	$CH_2(OH).CH_2.CH_2.SH$	581	$CH_3.CH(SH).CH(SH).CH_2OH$
264	$CH_2(SH).CH(OH).CH_3$	584	$CH_2(SH).CH(SH).CH_2.S.CH_2.CH(SH).CH_3$
271	$CH_2(OH).CH(OH).CH_2.SH$	614	$CH_2(SH).CH(O.CO.CH_3).CH_2SH$
449	$CH_2(SH).CH(SH).CH_2.OC_2H_5$	622	$CH_2(SH).CH_2.CH(SH).CH_2O.CO.CH_3$
458	$CH_2(SH).CH(SH).CO.OCH_3$	633	$C(CH_2.SH)_2(OH)(CO_2H)$
476	$CH_2(SH).CH(SH).CH(OCH_3).CH_2.OCH_3$	639	$CH_2(OH).CH(SH).CH(SH).CH_2OH$
484	$CH_2(SH).CH(SH).CO_2H$	713	$CH_2(SH).CH(SH).CH_2.O.C_6H_5$
493	$CH_2(SH).CH(OH).CH(SH).CH_3$	735	
496	$CH_2(SH).C(CH_3)(OH).CH_2.SH$	756	
515	$CH_2(SH).CH_2.CH(SH).CH_2.OH$	765	$CH_2(SH).CH(OH).CH(SH).CH_2SH$
522	$CH_2(SH).CH(SH).CH(OH).CH_3$		
552	$CH_3.CH(SH).CH_2.S.CH_2.CH_2OH$		

TABLE II

ACTIVE THIOLS

All compounds were administered subcutaneously in arachis oil.

B.R.L. No.	Formula	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
—	Dimercaprol $CH_2(SH).CH(SH).CH_2OH$	50·0	100	9·5	7·0	—	—
479	$CH_2(SH).CH(SH).CH_2Cl$	6·25	100	—	24·0	16·0	9·0
489	$CH_2(SH).CH(SH).CH_2$ S $CH_2(OH).CH(SH).CH_2$	25–50	>500	—	14·0	8·0	3·5
550	$CH_3.CH(SH).CH_2.S.CH_2.CH_2SH$	>100·0	200	10·0	7·0	2·5	—
555	$CH_2(OH).CH(SH).CH_2$ $CH_3.CH(SH).CH_2.S$	—	250	7·5	4·0	2·0	—
567	$CH_2(SH).CH_2(SH).CH(Cl).CH_3$	50·0	500	11·0	7·0	3·5	—
578	$CH_2(SH).C(CH_3)(Cl).CH_2SH$	>50·0	400	—	8·0	6·0	—
609	$CH_3.CH(SH).CH(SH).CH_2Cl$	>50·0	300	—	4·0	6·0	—
784	$CH_2(SH).[CH_2]_8.CH(SH).CH_2OH$	>50·0	500	—	4·0	0·5	—

as an infected control. Some compounds were tested for oral activity, either by administration by stomach tube or in the diet. The doses administered were again 1/2, 1/5, 1/10, and 1/20th of their approximate acute oral LD50. The water-soluble compounds were given in aqueous solution in a volume of 0.2 ml./20 g. and the insoluble solids as acacia suspension, 0.2 ml./20 g. When the compounds were given in the diet the solution or suspension was added to 250 g., so that the appropriate dose was contained in 5 g., that is, the average daily intake for one mouse. Daily dosing commenced in all cases 24 hr. after infection and continued until the twentieth day of the experiment. The TD50 values (median survival time) were determined by plotting the accumulative percentage mortality against time in days on log probit paper, and in cases where there was an increase in TD50 value over that of the control the results were analysed by the method of Litchfield (1949).

Toxicities.—Only approximate estimates of the acute LD50 values were obtained. The compounds were prepared as oil and water emulsions using acacia as the dispersing agent, and injected subcutaneously into groups of 5 mice per dose level.

RESULTS

Thiols

The inactive thiols are shown in Table I and the active thiols in Table II. The parent compound of the series, dimercaprol (2,3-dimercaptopropanol), and all the active thiols, apart from 2,3-dimercaptopropyl chloride (B.R.L. 479), were only slightly active *in vivo*. *In vitro* their activity was negligible. The compounds which were active possessed free thiol groups and a hydroxyl group, but not necessarily all compounds of this type were active. Acylation of the hydroxyl group or replacement of the hydroxyl group with a thiol, carboxyl or substituted carboxyl group abolished activity. Compounds having only one thiol group were inactive. If the hydroxyl group in the compounds having two vicinal thiol groups was replaced by a chlorine atom, activity was retained and might in fact be enhanced, for example, 2,3-dimercaptopropyl chloride (B.R.L. 479) was appreciably active *in vivo*, giving a considerable extension of the survival time. *In vitro* it had an

activity of 6.25 μg./ml., being the only member of this group which had a significant *in vitro* activity.

The chloro compounds B.R.L. 479, B.R.L. 567 and B.R.L. 609, which were prepared from the hydroxyl analogues and hydrochloric acid, may be contaminated with isomeric structures (Doyle, Holland, Mansford, Nayler, and Queen, 1960).

Dithiolans

Ring closure to form dithiolans resulted in complete loss of activity—Table III.

Thiol Esters

The inactive thiol esters are listed in Table IV and the active thiol esters in Table V. Some of the active compounds showed considerable *in vitro* activity. 2,3-Di(acetylthio)propyl acetate (B.R.L. 403) and 2,3-di(acetylthio)propanol (B.R.L. 464) are active at a concentration of 0.8 μg./ml. 1,2,3-Tri(acetylthiopropane (B.R.L. 411) was also very active *in vitro*. These three compounds also showed good activity *in vivo*. Increasing the size of the acyl group to a butyryl group, however, led to a considerable decrease both of *in vivo* and *in vitro* activity. The substitution of other radicals also led to a diminished activity.

TABLE III

DITHIOLANS

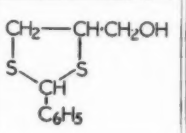
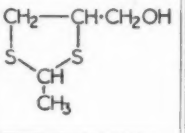
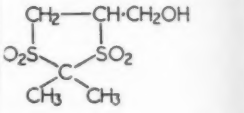
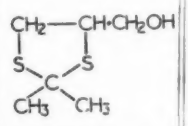
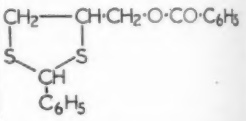
B.R.L. No.	Formula	B.R.L. No.	Formula
401		613	Glucose derivative of dimercaprol
412		647	
414		650	

TABLE IV

INACTIVE THIOL ESTERS

B.R.L. No.	Formula
450	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).CH_2.S.CO.CH_3$
471	$CH_2(O.CH_2COOH)CH(S.CO.CH_3).CH_2S.CO.CH_3$
543	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH_2.CH_2.S.CO.CH_3$
545	$CH_3CH(O.CO.CH_3).CH(S.CO.CH_3).CH_2.S.CO.CH_3$
568	$CH_2(S.CO.CH_3).C(CH_3)(O.CO.CH_3).CH_2.S.CO.CH_3$
583	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).CH_2.CH_2.S.CO.CH_3$
585	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH(S.CO.CH_3).CH_3$
640	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH(S.CO.CH_3).CH_2.O.CO.CH_3$
657	$C(CH_2.S.CO.CH_3)_2(O.CO.CH_3)(CO_2H)$
755	$CH_3(S.CO.CH_3).CH(O.CO.CH_3).[CH_2]_4.S.CO.CH_3$
766	$CH_2(S.CO.CH_3).CH(S.CO.CH_3).CH(O.CO.CH_3).CH_2.S.CO.CH_3$
788	$CH_2(S.CO.CH_3).[CH_2]_8.CH(S.CO.CH_3).CH_2O.CO.CH_3$
826	$CH_2(S.CH_3).CH_2.S.CO.CH_3$

TABLE V

ACTIVE THIOL ESTERS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous suspension.

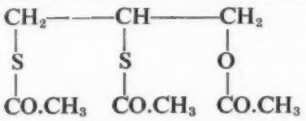
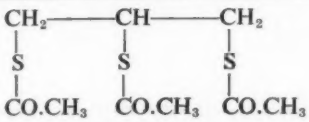
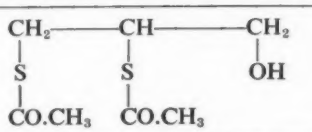
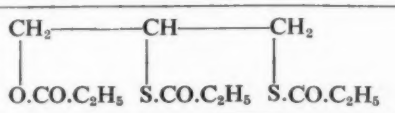
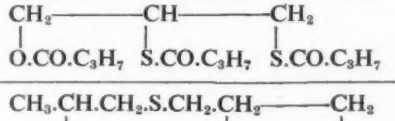
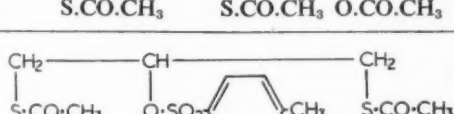
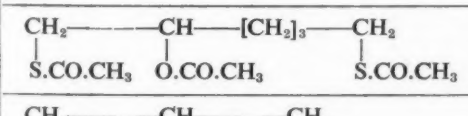
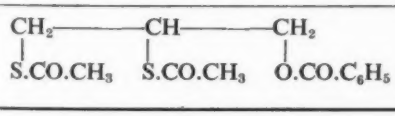
B.R.L. No.	Formula	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50		
				1/2	1/5	1/10
403		0·8	200	15·25	9·0	2·5
411		1·6	99	17·5	9·5	2·5
464		0·8	150	11·5	4·5	2·75
535		—	>500	15·0	2·0	2·0
536		6·25	500	2·0	−0·5	−0·5
562		6·25	500	2·5	−1·0	−1·5
655		6·25–12·5	>500	—	14·0	11·5
			150 (a)	—	2·0	3·0 (a)
758		>50·0	>500	—	4·0	1·5
877		—	>500	—	4·0	2·5

TABLE VI

INACTIVE DIMERCAPTOPROPYL ESTERS

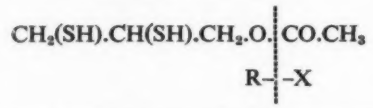
B.R.L. No.	Formula
646	$CH_2(SH).CH(SH).CH_2.O.CO.[CH_2]_{14}.CH_3$
694	

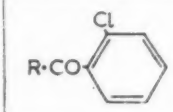
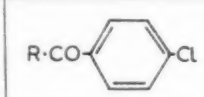
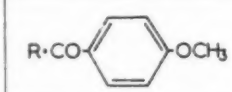
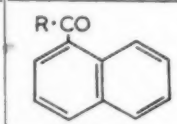
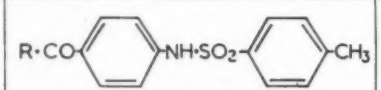
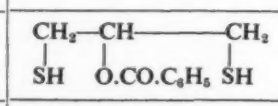
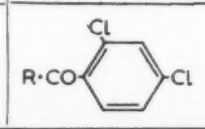
TABLE VII

ACTIVE DIMERCAPTOPROPYL ESTERS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous suspension, (*b*) orally in arachis oil.

General formula:



B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
463	R–CO.CH_3	3·125–6·25	78	15·0	8·0	4·4	—
504	R–CO.C_2H_5	12·5	150	13·0	4·0	—	—
510	R–CO.C_3H_7	25·0	400	13·0	8·5	1·5	—
587	R–CO.C_6H_5	12·5	>500	28·4	18·6	16·4	9·0
			600 (a)	5·2	2·5	1·25 (a)	—
			—	−3·5	11·5	0 (b)	—
636		25·0	>500	20·0	29·0	22·8	10·7
			500 (a)	6·5	2·0	2·5 (a)	—
637		12·5	500	54·0	29·0	16·0	11·25
638	R–CO.CH_2.C_6H_5	25·0	500	—	14·5	8·5	1·0
			>500 (a)	—	3·0	3·5 (a)	—
651		25·0	200	22·5	11·5	5·5	—
668		50·0	>500	—	9·5	2·5	—
670		>50·0	>500	—	8·0	0·5	—
671	R–CO.CH_2.S.C_2H_5	25·0	300		8·0	3·5	—
673	R–CO.CH_2.S.CH_2.C_6H_5	50·0	>500	14·0	10·5	5·0	--
			>500 (a)	3·0	1·0	−0·5 (a)	--
680		1·56	500	34·0	19·0	10·25	3·5
693	R–CO.$[CH_2]_2$.S.CH_3	25·0	500	—	8·5	6·0	—
707		25–50	150	—	3·0	0·75	--

Dimercaptopropyl Esters

The inactive compounds are given in Table VI and the active compounds in Table VII. When the hydroxyl group is esterified there is usually a considerable increase in antitubercular activity, particularly in compounds with vicinal thiol groups, and where the hydroxyl group is adjacent to these. 2,3-Dimercaptopropyl benzoate (B.R.L. 587), 2,3-dimercaptopropyl *o*-chlorobenzoate (B.R.L. 636) and 2,3-dimercaptopropyl *p*-chlorobenzoate (B.R.L. 637) are the most active of this particular type. The precise nature of the ester radical, however, does not seem to be of great importance in determining the degree of activity. All of the *O*-esters of dimercaprol were, however, comparatively inactive *in vitro*.

A notable exception to the general pattern is 2-mercapto-1-mercaptomethylethyl benzoate (B.R.L. 680), which has the two thiol groups separated by the ester group. This compound is active both *in vitro* and *in vivo*. The isomer 2,3-dimercaptopropyl benzoate (B.R.L. 587) is relatively inactive *in vitro*.

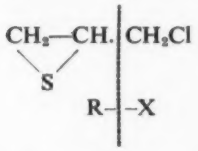
Episulphides

By eliminating the elements of water from thiol compounds containing a vicinal hydroxyl group

TABLE VIII

INACTIVE EPISULPHIDES

General formula:



B.R.L. No.	R.X
509	$R.CH_3$
511	$R.CH_2Cl$
512	$R.CH_2.O.C_2H_5$
513	$R.CH_2.S.C_2H_5$
514	$R.CH_2.N(C_2H_5)_2$
520	$R.CH_2.S.CO.NH.C_6H_5$
723	$R.CH_2.S.CH_3$

TABLE IX

ACTIVE EPISULPHIDES

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous solution, (b) orally in arachis oil, (c) in the diet.

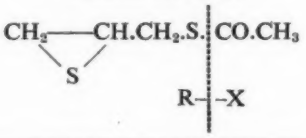
B.R.L. No.	Formula ($R=CH_2—CH—$, S)	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
482	$R.CH_2SH$	12·5	44 88 (a)	−1·5	−3·0	−3·0 (c)	—
				17·2	17·0	14·5	4·0
				3·0	1·25	1·0 (a)	—
				5·5	−1·0 (b)	—	—
528	$CH_3.CH(S)CH.CH_2SH$	100	200	—	2·5	1·5	—
553	$R.CH_2.S.S.CH_2.R$	—	500	3·5	18·0	14·0	—
577	$CH_2(S)CH.CH_2.CH_2SH$	50·0	400	—	4·5	6·75	—
599	$R.CH(SH).CH_3$	12·5–25·0	100	2·5	1·5	−1·0	—

TABLE X

EPISULPHIDES WITH *S*-ACYL GROUPS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (a) orally in aqueous suspension, (b) orally in arachis oil, (c) in the diet, (d) orally in acacia suspension, (e) subcutaneously in acacia suspension.

General formula:



B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
459	$R.CO.CH_3$	0·4–0·8	45	17·25	14·0	12·0	4·5
			110 (a)	9·25	6·25	0·5 (a)	—
				3·5	2·0 (b)	—	—
				−2·5	−1·5	−2·5 (c)	—
540	$R.CO.C_2H_5$	1·56	100	21·0	17·5	11·0	—
546	$CH_2(S)CH.CH_2.CH_2.S.CO.CH_3$	—	300	14·5	5·25	0·5	—
561	$R.CO.C_3H_7$	6·25–12·5	100	14·5	13·5	7·0	—
571	$CH_2(S)C(CH_3).CH_2.S.CO.CH_3$	50·0	300	2·5	2·7	1·5	—
580	$R.CO.CH_2.CH_2CO_2H$	25·0	100	— 7·5	11·5 3·5	5·0 4·0	—
			200 (a)	—	1·5	1·5 (a)	—
590	$R.CO.CH_2Cl$	25·0	60	16·0	17·5	16·3	6·0
			150 (a)	3·5	2·5	2·5 (a)	—
595	$CH_2(S)CH.CH(S.CO.CH_3).CH_3$	25–50	300	5·5	3·5	1·5	—
600	$R.CO.[CH_2]_2.CO.OCH_3$	1·56–3·125	100	17·5	6·5	12·0	—
601	$CH_2(S)CH.[CH_2]_3.S.CO.CH_3$	3·125	>500	17·5	10·0	1·5	—
602	$R.CO.O.C_2H_5$	1·56	150	16·5	18·0	15·0	4·5
605	$R.CO.C_6H_5$	1·56	300	30·5	16·5	20·5	0
			400 (a)	4·0	2·5	2·0 (a)	—
615	$R.CO.CH_2.CO.O.C_2H_5$	3·125	75	22·5	11·0	8·0	—
617	$R.CO.CHCl_2$	25·0	150	—	17·5	10·0	5·0

TABLE X—*continued.*

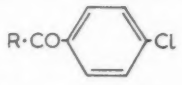
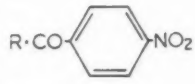
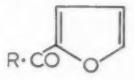
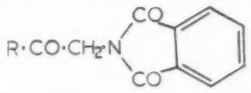
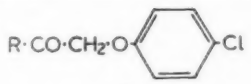
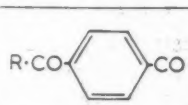
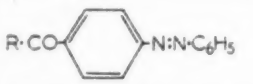
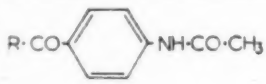
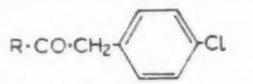
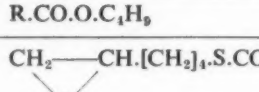
B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg/kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
625	R.CO.CH_2.CH_2Cl	6·25	300	—	19·5	17·5	—
629	R.CO.CH_2.C_6H_5	12·5	500	22·5	27·5	17·0	11·0
			>500 (a)	13·0	9·0	6·0 (a)	—
			—	—	4·0	0·5 (b)	—
635	R.CO.CCl_3	25·0	500	—	—	22·5	21·5
643	R.CO.CO.O.C_2H_5	12·5	100	17·5	13·5	10·5	—
649		50·0	200	29·5	12·5	2·5	—
			>1000 (d)	10·0	1·5	0·5 (d)	—
656		6·25	>500	29·5	15·0	11·0	—
			>500 (d)	16·25	5·2	2·75(d)	—
658		0·39	300	—	28·5	26·3	8·5
			500 (a)	—	7·0	0·5 (a)	—
664		>50·0	300	15·0	16·0	15·0	—
			1000 (d)	4·0	2·0	3·0 (d)	—
669		>50·0	200	—	5·5	2·5	—
672	R.CO.$CH(C_6H_5)_2$	>50·0	>500	6·0	1·0	1·0	—
677	R.CO.CH_2.S.C_2H_5	6·25	100		18·5	9·0	4·0
681	R.CO.$[CH_2]_{14}$.CH_3	>50·0	>500	4·0	0·5	−1·0	—
690	R.CO.CH_2.NH.CO.O.CH_2.C_6H_5	25·0	150	12·5	8·5	1·5	—
710	R.CO.CH.$[CH_2]_4$.CH_2	6·25–12·5	>500	—	34·5	23·5	1·5
711	R.CO.$[CH_2]_2$.CO.CH_3	25·0	100	—	23·5	—	—
			200 (a)	—	7·0	2·5 (a)	—
			—	—	2·5	2·0 (b)	—
721		3·125	>500		25·0	17·0	17·0 (e)

TABLE X—*continued.*

B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg/kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
727	$R.CO.CH_2.NH.CO.C_6H_5$	>50·0	400		17·0	16·0	1·5 (e)
728		3·125	>500	—	8·0	11·0	2·0 (e)
733		3·125–6·25	>500	—	21·0	13·0	5·5 (e)
746	$R.CO.O.CH_3$	6·25–12·5	50	—	8·5	4·5	—
747		25·0		—	12·5	9·0 (e)	—
748	$R.CO.CH(Cl).C_6H_5$	25·0	>500	—	2·5	0·5 (e)	—
753	$R.CO.O.C_4H_9$	3·125	>500	—	22·0	8·0	—
785		—	300	—	2·0	2·0	—

to form episulphides the compounds produced have enhanced antitubercular activity. The inactive compounds are shown in Table VIII and the active compounds in Tables IX and X. Since the synthetic method (Doyle, Holland, Mansford, Nayler, and Queen, *J. chem. Soc.*, in the press) was not structurally definite either or both of the preparations, B.R.L. 528 and B.R.L. 599, may be mixtures of the two isomeric structures. Of the compounds in Table IX only 3-mercaptopropylene sulphide (B.R.L. 482) and di(2,3-epithiopropyl) disulphide (B.R.L. 553) show good activity. On the other hand, the majority of episulphides with *S*-acyl groups (Table X) were highly effective chemotherapeutic agents. The compounds with an episulphide ring must, however, be comparatively simple in order to be active ; compounds with high activity being confined to *S*-acyl derivatives of 3-mercaptopropylene sulphide (B.R.L. 482). Substitution of methyl groups on any of the carbon atoms almost abolishes activity. The addition of groups other than simple acyl radicals also abolishes activity. The nature of the acyl radical, on the condition that it is a simple group, would not appear to be the main determining factor for antitubercular activity, but the acetyl (B.R.L. 459) and furoyl (B.R.L. 658) derivatives both have high *in vivo* and *in vitro* activities.

DISCUSSION

A large number of thiol compounds and related derivatives have been investigated for antitubercular activity, and many have proved to be active *in vivo*. There are, however, a number of discrepancies between the *in vitro* activity and the *in vivo* activity, and an explanation for this must be sought.

Since the majority of the episulphides are very active *in vivo*, it would appear that the episulphide structure is the necessary moiety for antitubercular activity. This view is supported by the investigations of Miles and Owen (1952) and Harding and Owen (1954), who have shown that acetylated thiols with a vicinal hydroxyl group in the presence of mild alkali are deacetylated with accompanying ring closure to form episulphides. The activity of the thiols and thiol *O*-esters can therefore be accounted for by such a mechanism. Further confirmation that an active derivative is formed has been obtained by the findings of Mansford and

Langley (to be published), who have shown that the addition of 3-acetylthiopropylene sulphide (B.R.L. 459) to serum leads to the formation of 3-mercaptopropylene sulphide (B.R.L. 482). If B.R.L. 482 is the active derivative, the absence of activity *in vitro* may be explained by the fact that it is an unstable compound and under the mild alkaline conditions of the *in vitro* test it forms inactive polymers.

The activity of the compounds tested is not due to their conversion to ethanethiol which has been shown to be the active agent produced by other thiol antitubercular agents (Oginsky, Solotorovsky, and Brown, 1955, 1956). We have been unable to detect ethanethiol in the expired air of animals injected with any of the compounds tested, nor has it been possible by using B.R.L. 587 containing radioactive ^{35}S to detect ^{35}S in the breath. Over 80% of the injected ^{35}S was recovered in the urine of rats in 24 hr. as sulphate.

The activity of the compounds which are active *in vitro* can possibly be explained by the assumption that the compound is absorbed on the surface of the organisms and the active metabolite (i.e., B.R.L. 482) is formed in close proximity to it and is therefore able to exert an antibacterial action before it is destroyed chemically. While there is little evidence for this theory, it does provide a likely explanation.

This explanation could also account for the apparent discrepancy between activities of 2,3-dimercaptopropyl benzoate (B.R.L. 587) and 1,3-dimercapto-2-propyl benzoate (B.R.L. 680) *in vitro* and *in vivo*. It is possible that B.R.L. 680 can be readily converted *in vitro* to the parent episulphide whereas B.R.L. 587 cannot. *In vivo*, however, the reaction may readily take place, with the result that both compounds are equally active. It is unlikely that the compounds are simply dealkylated to the hydroxyl compound without ring closure taking place, since the parent hydroxyl compound (B.R.L. 231) of B.R.L. 680 is highly toxic and completely inactive *in vivo*.

We wish to thank Mr. F. P. Doyle and his colleagues for preparing the compounds; Dr. L. N. Owen for the supply of 3,4-dimethoxybutane-1:2-dithiol (B.R.L. 476), 1,4-dihydroxybutane-2:3-dithiol (B.R.L. 639) and 1,4-diacetoxy-2,3-(diacetylthio)butane (B.R.L. 640); Dr. J. H. C. Nayler for guidance on chemical nomenclature; and Mr. D. Wright for technical assistance.

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THE ANTITUBERCULAR ACTIVITY OF 3-ACETYLTHIOPROPYLENE SULPHIDE AND 3-(2-FUROYLTHIO)PROPYLENE SULPHIDE

BY

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Two derivatives of 3-mercaptopropylene sulphide (B.R.L. 482), 3-acetylthiopropylene sulphide (B.R.L. 459) and 3-(2-furoylthio)propylene sulphide (B.R.L. 658), have been tested in mice for antitubercular activity. The compounds were administered daily in arachis oil by the subcutaneous route. When tested by a prolongation in survival time B.R.L. 459 was less active than streptomycin, while B.R.L. 658 was of the same order of activity. When assessed by the lesions present in the lungs after 14 days' treatment B.R.L. 658 was as active as isoniazid and B.R.L. 459 was of the same order of activity as streptomycin. Neither compound, however, cured an established infection and resistance developed rapidly to both compounds, both *in vitro* and *in vivo*. It is concluded that, in view of their adverse physical and pharmacological properties, the compounds are unsuitable for clinical use.

Acred and Brown (1960) have tested a series of new antitubercular compounds related to dimercaprol (2,3-dimercaptopropanol) and have produced evidence that the activity of the compounds depends on the formation of 3-mercaptopropylene sulphide. *S*-acyl derivatives of this compound possess considerable antitubercular activity. Two of the most active compounds, 3-acetylthiopropylene sulphide (B.R.L. 459) and 3-(2-furoylthio)propylene sulphide (B.R.L. 658), have been examined in greater detail.

METHODS

The methods and techniques for assessing antitubercular activity *in vitro* and *in vivo* were the same as those used by Acred and Brown in the previous paper. The organism employed in all tests was the human strain ($H_{37}Rv$) of *Mycobacterium tuberculosis*. In all tests the propylene sulphides were administered in arachis oil solution; streptomycin and isoniazid were given in aqueous solution.

Determination of the Extension of the Median Survival Time (TD50).—The antibacterial activity of the compounds was tested in mice (18 to 22 g.) infected with *Mycobacterium tuberculosis* and compared with that of streptomycin and isoniazid. Doses of one-fifth, one-tenth, one-twentieth and one-fortieth of the acute subcutaneous LD50 values were administered daily by the subcutaneous route for 20 days following the infection; the activity being expressed as the extension in days of the median survival time, over that of the infected control.

Lung Lesion Test.—As a further check on activity the procedure described by Solotorovsky, Winsten, Ironson, Brown, and Becker (1954) was employed. Ten mice (18 to 22 g.) were allocated to each group. The mice were infected intravenously with 0.8 mg. wet weight of *Mycobacterium tuberculosis*. The compounds were administered at one-fifth, one-tenth, one-twentieth and one-fortieth of the acute subcutaneous LD50 values. Treatment was stopped when 50% of the infected control animals died. On that day all surviving animals were killed and the lungs removed and kept in 10% formol saline for 48 hr. The degree of lung tuberculous involvement was scored in percentage for each group, the final result being expressed as the mean percentage tuberculous involvement.

Activity Against an Established Infection.—Groups of 10 mice were infected intravenously with 0.8 mg. wet weight *Mycobacterium tuberculosis*. Treatment was commenced after seven days, and continued until the twentieth day. Doses of the propylene sulphides equal to one-fifth and of streptomycin equal to one-tenth the acute subcutaneous LD50 were given by the subcutaneous route. The TD50 values were estimated and compared with an infected control group.

In vivo *Resistance.*—The propylene sulphides and streptomycin were administered daily by the subcutaneous route at one-twentieth of their acute subcutaneous LD50 values. Groups of 10 mice (18 to 22 g.) infected intravenously with 0.8 mg. wet weight *Mycobacterium tuberculosis* were used. When two or three mice remained in each group they were killed, and the lungs and spleens removed and homogenized. The tubercle bacilli were cultured from the

homogenates on Löwenstein-Jensen slopes and in Proskauer and Beck medium. For the next passage a further group of mice were infected with an inoculum prepared from the isolated organism grown on Proskauer and Beck medium. The *in vitro* sensitivity and virulence of the bacilli isolated at the first, third, and fifth passages were determined and compared with the results obtained for bacilli isolated at each passage taken from an infected, untreated control group of mice. The *in vitro* sensitivity was determined in Dubos medium. The virulence to mice was checked by infecting intravenously a group of 10 mice with 0.8 mg. wet weight of the organism isolated, and determining the TD50 in the absence of drug treatment.

Development of Resistance in vitro.—A suspension of a 10-day culture of *Mycobacterium tuberculosis*, standardized to Wellcome opacity tube 2, was diluted 1:10 with Dubos medium. Two drops of this suspension were added to tubes, each containing 2.5 ml. of serial dilutions of B.R.L. 459 and B.R.L. 658, streptomycin, and isoniazid in Dubos medium. Incubation was carried out at 37° for 7 days. The concentration of drug in μg./ml. which just inhibited growth (minimal inhibitory concentration) was noted. The inoculum for the subsequent passage was taken after a further 7 days' incubation from the tube containing the highest concentration of drug in which growth occurred. Passaging was continued until a high degree of resistance to the compounds had developed.

Results

Extension of Median Survival Time.—The results obtained are given in Table I. B.R.L. 459 is less active than streptomycin at all dose levels. B.R.L. 658 also appeared to be less active, but in 1 experiment with streptomycin a rather unusually long extension of the TD50 was recorded. If this test is not taken into account the activity of B.R.L. 658 and streptomycin would be of the same order. Isoniazid is considerably more active than both the compounds under test.

Lung Lesion Test.—The results obtained are given in Table II. When tested in this way B.R.L. 459 was of the same order of activity as streptomycin. At the dose levels employed all the lungs showed advanced tuberculous involvement. B.R.L. 658, on the other hand, was more active than streptomycin. At one-tenth of the subcutaneous LD50 dose the tuberculous involvement was slightly greater than minimal, while at one-twentieth of the LD50 there was only an average of 25% involvement. However, at one-fortieth of

Table I

THE ANTITUBERCULAR ACTIVITY OF 3-ACETYLTHIOPROPYLENE SULPHIDE (B.R.L. 459) AND 3-(2-FUROYLTHIO)PROPYLENE SULPHIDE (B.R.L. 658), STREPTOMYCIN AND ISONIAZID IN MICE

The extension of the TD50 value is the increase in survival time of the treated mice over the untreated mice, measured from the regression of percentage mortality against time on log-probit paper at the 50% mortality level. The *in vitro* activity was determined in Dubos medium seven days after inoculation.

Compound	Subcutaneous LD50 mg./kg.	*In vitro* Activity (M.I.C.) μg./ml.	Expt. No.	Extension in TD50 over Control—Days. Dose Fraction of Subcutaneous LD50			
				1/5	1/10	1/20	1/40
B.R.L. 459 ..	45·0	0·4–0·8	1		13·0	1·0	1·0
			2		7·0	2·5	2·5
			3		2·5	2·5	0
			4	14·0	12·0	4·5	0
B.R.L. 658 ..	300·0	0·8	1		17·0	9·0	3·0
			2		7·5	5·5	5·5
			3		4·5	0	1·0
			4	28·5	26·5	8·5	—
Streptomycin ..	520·0	0·4	1	—	16·5	9·0	4·0
			2	—	16·0	7·0	7·0
			3	59·0	38·0	15·0	6·5
			4	12·5	10·0	9·0	5·5
Isoniazid ..	182·0	0·05	1	—	89·0	44·0	38·0
			2			70·0	75·0

TABLE II

LUNG LESION TEST

The mice were killed and the lungs examined on the day 50% of the control mice died.

Compound	Expt. No.	% Lung Tuberculous Involvement. Dose Fraction of Subcutaneous LD50		
		1/10	1/20	1/40
B.R.L. 459	1	57	79	71
	2	100	100	100
	3	72	82	100
	Mean	76·3	87·0	90·3
B.R.L. 658	1	12	37	92
	2	8	27	50
	3	16	12	83
	Mean	12	25	75
Streptomycin	1		100	
	2		86	
	3		100	
	4		100	
	5	66	90	
	6	57	90	
	Mean	61·5	94·3	
Isoniazid	1	0		
	2	5	50	

the LD50 there was advanced involvement, but this was not a great deal worse than that seen in mice treated with one-tenth the subcutaneous LD50 of streptomycin. From the limited data with isoniazid, B.R.L. 658 appeared to have the same order of activity.

TABLE III

DELAYED THERAPY

Compound	TD50—Days. Dose Fraction of Subcutaneous LD50	
	1/5	1/10
B.R.L. 459	21·0	
B.R.L. 658	20·5	
Streptomycin ..		100% survival after 30 days
Infected control (no treatment)	20·0	

Action Against Established Infection.—Neither B.R.L. 658 nor B.R.L. 459 gave any protection to mice which had been infected 7 days previously. On the other hand, streptomycin had a pronounced antitubercular action ; all the mice still survived 30 days after the infection (see Table III).

In vivo *Resistance.*—The *in vitro* sensitivities of the bacilli isolated from mice at the end of the first, third and fifth passages are given in Table IV.

TABLE IV

VIRULENCE AND *IN VITRO* SENSITIVITIES OF *MYCOBACTERIUM TUBERCULOSIS* PASSAGED IN MICE

Mice infected with *Mycobacterium tuberculosis* were treated daily with one-twentieth the subcutaneous LD50 of B.R.L. 459, B.R.L. 658, and streptomycin for twenty days. At the end of each passage the organism was isolated on Löwenstein and Jensen slopes from the mice and the *in vitro* sensitivity (minimum inhibitory concentration—μg./ml.) determined in Dubos medium. The virulence was determined by infecting groups of ten mice and estimating the TD50 in the usual manner.

Passage	1		3		5	
Compound	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days
B.R.L. 459	0·78	19·0	1·56–3·13	18·0	12·5	12·0
Control ..	1·56	18·0	—	13·0	0·78	15·0
B.R.L. 658	0·78	22·0	6·25	20·0	3·13	14·0
Control ..	0·39	18·0	—	13·0	0·39	15·0
Streptomycin	0·78	—	0·39	14·0	0·2	19·0
Control ..	0·78	18·0	—	13·0	0·2	15·0

Resistance of the bacilli to B.R.L. 459 and B.R.L. 658 had developed markedly by the end of the fifth passage, while no change in the sensitivity to streptomycin was detectable. In every instance the virulence of all the isolated bacilli was fully maintained.

In vitro *Resistance.*—Resistance induced by *in vitro* transfer to B.R.L. 658 and B.R.L. 459 developed with exceptional rapidity (see Fig. 1). The minimal inhibitory concentration at the commencement of the test for B.R.L. 658 and B.R.L. 459 was 0.39 μg./ml., and within five passages this

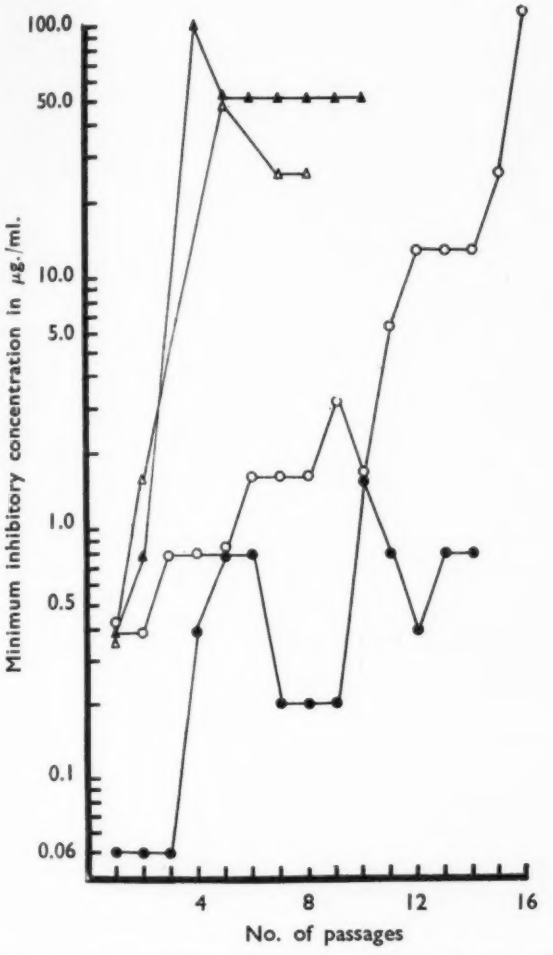

FIG. 1.—Development of resistance of *Mycobacterium tuberculosis* ($H_{37}Rv$) in Dubos medium to B.R.L. 459, B.R.L. 658, streptomycin, and isoniazid. ▲ B.R.L. 459. △ B.R.L. 658. O Streptomycin. ● Isoniazid.

rose to 50 to 100 μg./ml. for B.R.L. 459, and 25 to 50 μg./ml. for B.R.L. 658. In the control series resistance to streptomycin began to develop within twelve passages, the minimal inhibitory concentration rising from 0.39 to 6.25 μg./ml. With isoniazid resistance began to develop at the fourth passage, but did not increase to the degree shown by the propylene sulphides.

DISCUSSION

B.R.L. 459 and B.R.L. 658 are highly active antitubercular compounds when tested in mice infected with the $H_{37}Rv$ strain of *Mycobacterium tuberculosis*. B.R.L. 658 is the more active of the two compounds, comparing favourably with streptomycin by the TD50 test, and is of the same order of activity as isoniazid by the lung lesion test. B.R.L. 459 shows a corresponding difference in relative activity to streptomycin and isoniazid by the two methods. The difference in relative activity observed using the two methods is probably due to the compounds having a bacteriostatic and not bactericidal action *in vivo*. In the TD50 test administration of the compounds is stopped after 20 days when a generalized lethal infection develops. On the other hand, in the lung lesion test the mice are still under treatment when they are killed. The growth of the organism is well suppressed at this stage and the lungs are practically devoid of lesions. The lung lesion test therefore gives a more favourable impression of relative activity.

Resistance to the compounds develops exceptionally rapidly *in vitro* and also *in vivo*. They are also incapable of curing an established infection and are ineffective orally. When administered daily over a period of six months to guinea-pigs infected with *Mycobacterium tuberculosis* they give rise to ulcers of such severity that they render an assessment of antitubercular activity in guinea-pigs impracticable. In addition, the compounds are odorous, oily, water-insoluble liquids which are unstable in the presence of water and in mild alkaline conditions. In spite, therefore, of their initial highly promising activity in mice, their adverse physical and pharmacological properties would seem to rule out their possible clinical use.

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INTERACTION BETWEEN HISTAMINE AND DICHLOROISOPROTERENOL, HEXAMETHONIUM, PEMPIDINE, AND DIPHENHYDRAMINE, IN NORMAL AND RESERPINE-TREATED HEART PREPARATIONS

BY

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Histamine stimulated the isolated auricles and heart of the guinea-pig. The effect was best seen in auricles which had been previously depressed by treatment with reserpine. Ganglionic blocking drugs (hexamethonium and pempidine), applied to auricles which had been previously treated with reserpine, abolished the diphasic effect of nicotine, but did not alter the response to histamine. Dichloroisoproterenol did not modify the stimulant action of histamine in isolated auricles, either before or after treatment with reserpine; nor did it alter the response of the isolated heart. Diphenhydramine reduced or blocked the stimulant action of histamine in auricles which had been previously treated with reserpine. The results support the hypothesis that histamine stimulates the myocardium by a direct action on specific receptors.

Histamine is known to stimulate the isolated heart of the rabbit and cat (Dale and Laidlaw, 1910; Gunn, 1926; Staub and Grossmann, 1930), of the guinea-pig (Went and Lissak, 1935; Penna, Illanes, Ubilla, and Mujca, 1959), and of the rat and frog (Went, Varga, Szucs, and Feher, 1952). It also stimulates isolated auricles of the rabbit (Dews and Graham, 1946) and initiates contractions in isolated strips of ox ventricle (Iwao, 1938).

The stimulant action of histamine on cardiac tissue may be direct on myocardial receptors or indirect through the local release of catecholamines (Truex, 1950; Kottegoda, 1953). Evidence suggesting a direct action was recently obtained by Giotti and Jngianna (1959) and Pepeu, Mannaioni, and Giotti (1959) in experiments with isolated auricles which had been previously stopped by treatment with reserpine. Histamine, adrenaline, and noradrenaline restored the activity of the auricles but nicotine and ephedrine did not, presumably because the auricles had been partially depleted of catecholamines (Giotti, 1954; Pepeu, Masi, and Giotti, 1959).

The introduction of 1-(3,4 dichlorophenyl)-2 isopropyl aminoethanol hydrochloride (dichloroisoproterenol) (Powell and Slater, 1958), a specific antagonist of the catecholamines on the heart (Moran and Perkins, 1958), provided an opportunity to re-investigate the problem of the site of action of histamine on the heart.

METHODS

Isolated guinea-pig auricles were suspended in 50 ml. oxygenated Tyrode solution at 29°, according to the method of Giotti (1954). The auricles were connected to an isotonic lever with magnification of 5; the load on the auricles was 1.5 g. Contractions were recorded on a smoked drum.

The isolated guinea-pig heart was prepared by the method of Langendorff (1895) and Porter (1898) and was suspended in a modified Langendorff apparatus. The perfusion pressure was kept constant at 40 cm. water. The *p*H of the Ringer-Locke solution was 8.2 and of the coronary effluent 7.5. The temperature was adjusted to 38°. The apex of the ventricles was connected to an isotonic lever, which wrote on a smoked drum. The heart rate was taken from a dipolar surface electrogram. The coronary outflow was followed with the recording system of Giotti and Beani (1957).

The drugs used were: (−)-adrenaline bitartrate and (−)-noradrenaline bitartrate (Recordati); nicotine as supplied by the Italian State Monopoly of Tobacco, and crystallized by us as the bitartrate;

TABLE I

EFFECT OF DICHLOROISOPROTERENOL ON THE RESPONSE OF ISOLATED GUINEA-PIG AURICLES TO STIMULANT DRUGS

The experiments were carried out on the same preparations. Dichloroisoproterenol (10 μg./ml.) was allowed to act for 30 min. Each value is the mean±S.D. of 6 experiments.

Treatment of Auricles	Drug	Dose μg./ml.	Contraction Maximum Height in mm.		Rate per min.	
			Before Dose	After Dose	Before Dose	After Dose
Nil	Adrenaline	1	37·3±6	49·6±11	87±5	98±5
Dichloroisoproterenol ..	,,	1	14·5±6	17·6±2	83±5	83±5
Nil	Noradrenaline	1	37·5±6	49·8±11	86±4	96±2
Dichloroisoproterenol ..	,,	1	15·1±5	16·1±4	83±4	83±4
Nil	Histamine	2	37·7±6	47·2±8	89±4	95±3
Dichloroisoproterenol ..	,,	2	13·5±7	30·6±7	82±4	88±5

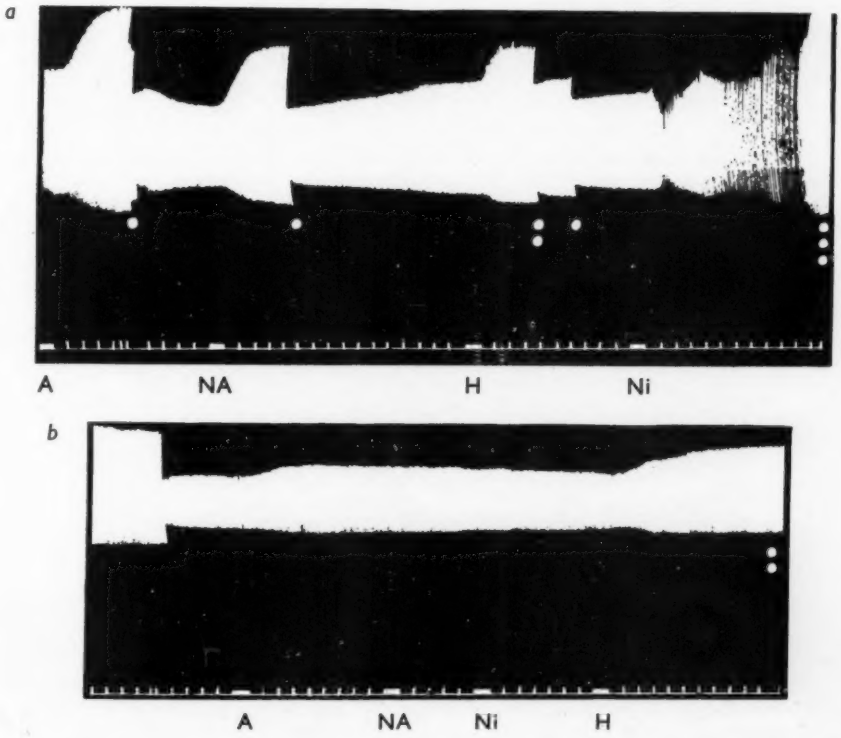

FIG. 1.—Dichloroisoproterenol 1×10^{-4} prevents the effects of the catecholamines and nicotine in guinea-pig isolated auricles; the effect of histamine is still present. (*a*) The effect of adrenaline 1×10^{-7} (*A*), noradrenaline 1×10^{-7} (*NA*), nicotine 5×10^{-5} (*Ni*), and histamine 1×10^{-6} (*H*). (*b*) The effect of the same substances 60 min. after treatment with dichloroisoproterenol. Upper tracing: height of contraction. Lower tracing: time, 10 sec. The washings are indicated by the white dots.

TABLE II

EFFECT OF RESERPINE AND DICHLOROISOPROTERENOL ON THE RESPONSE OF ISOLATED GUINEA-PIG AURICLES TO STIMULANT DRUGS

Reserpine (5 μg./ml.) was allowed to act for 5 hr.; dichloroisoproterenol (1 μg./ml.) for 10 min. before applying the stimulant drug. Experiments were carried out on the same preparations. Each value is the mean ± S.D. of 6 experiments.

Treatment of Auricles	Drug	Dose μg./ml.	Contraction Maximum Height in mm.		Rate per min.	
			Before Dose	After Dose	Before Dose	After Dose
Reserpine	Adrenaline	1	2·4±2	39·8±6	19·2±19	75·8±16
,, +dichloroisoproterenol	,,	1	0·8±1	5±5	6·6±8	11·4±13
Reserpine	Histamine	2	3·2±4	22·4±9	14·4±15	70·2±11
,, +dichloroisoproterenol	,,	2	0·6±1	20·4±9	4·8±7	50·2±3

histamine dihydrochloride (Roche); reserpine was kindly supplied by Ciba Ltd., and used as the phosphate; pempidine (1,2,2,6,6,-pentamethylpiperidine) gratefully acknowedged as a gift from Dr. A. Banchetti, Istituto Gentili, Pisa; Benadryl (diphenhydramine) Parke Davis; dichloroisoproterenol kindly supplied by Dr. N. J. Giarman and Dr. G. Pepeu, of Yale University.

The drugs were dissolved in the appropriate physiological solution and concentrations (w/v) are expressed as the salt. Dose is quoted as the concentration of drug in the bath.

The stimulant drug was applied to the auricles in successive doses. When the response was maximal, the bath fluid was changed three times. The antagonist was then allowed to act on the auricles for a period of 30 to 60 min. before retesting the effect of the stimulant drugs. Reserpine, however, was left in contact with the auricles for 4 to 6 hr.

RESULTS

Effect of Histamine, Adrenaline, Noradrenaline, and Nicotine on Isolated Guinea-pig Auricles Before and After Treatment with Dichloroiso-

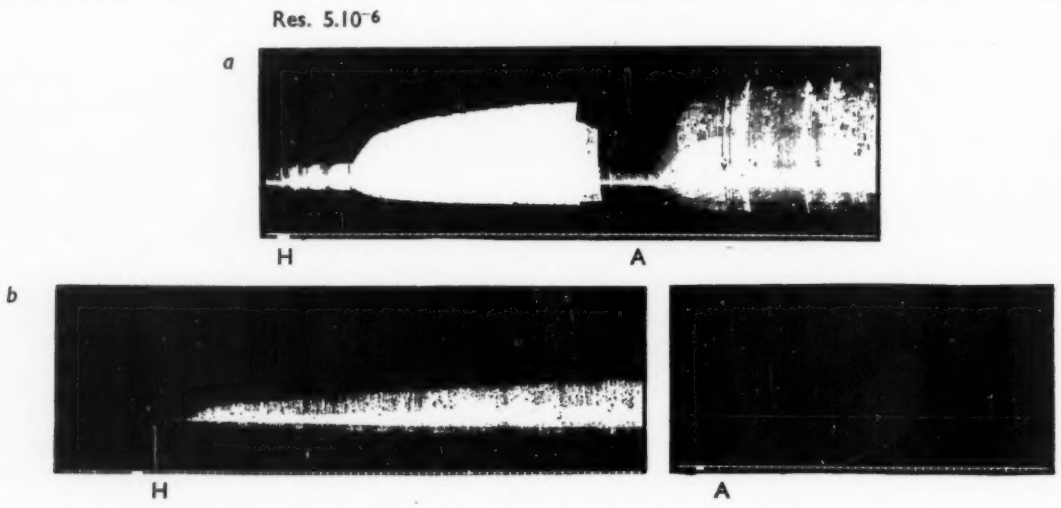

FIG. 2.—(*a*) Effects of histamine 1×10^{-6} and adrenaline 1×10^{-7} on guinea-pig auricles which had been previously treated with reserpine. (*b*) Effects of the same substances after treatment with dichloroisoproterenol 10^{-6} in addition to the previous treatment with reserpine. Upper tracing: height of contraction. Lower tracing: time, 10 sec.

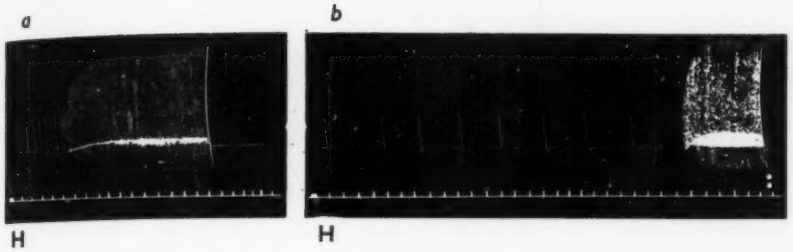

FIG. 3.—(*a*) Response of guinea-pig auricles, arrested by reserpine 5×10^{-6}, to histamine 1×10^{-6} (*H*). (*b*) Treatment with ganglionic-blocking agents (hexamethonium 1×10^{-4}) for 30 min. does not affect the histamine stimulation. Upper tracing: height of contraction. Lower tracing: time, 20 sec. The washings are indicated by the white dots.

proterenol (Table I, Fig. 1). — Dichloroisoproterenol (10 μg./ml.) abolished the stimulant effect of adrenaline (1 μg./ml.) and of noradrenaline (1 μg./ml.) on the rate, and greatly reduced the effect on the contraction, of the auricles. It also abolished the diphasic action (inhibition followed by stimulation) of nicotine (50 μg./ml.). Dichloroisoproterenol, however, did not abolish the response to histamine: the difference (17 mm.) between the mean for the contraction before the dose and the mean after the dose was significant ($P<0.05$).

Effect of Histamine and Adrenaline Before and After Dichloroisoproterenol on Isolated Guinea-pig Auricles Previously Treated with Reserpine (Table II, Fig. 2).—When dichloroisoproterenol (1 μg./ml.) was applied to auricles which had been previously treated with reserpine, the rate slowed and the contraction became smaller; in some experiments the auricles stopped. Under these conditions, the auricles

TABLE III

EFFECT OF ADRENALINE, NORADRENALINE, AND HISTAMINE ON THE ISOLATED HEART OF THE GUINEA-PIG, BEFORE AND AFTER TREATMENT WITH DICHLOROISOPROTERENOL

Experiments were carried out on the same preparations; dichloroisoproterenol was added to the perfusion fluid 30 min. before testing adrenaline, noradrenaline, and histamine. The values for the contraction are expressed as percentage increase of the height of the kymographic record. Each value is the mean ± S.D. of 6 experiments.

Stimulant Drug	Dose μg.	Percentage Increase in Contraction			
		Dichloroisoproterenol			
		Dose (μg./ml.): 0	1	5	10
Adrenaline	0·1	135±30	0		
		115±27		0	
		103±20			0
Noradrenaline ..	0·1	129±6	0		
		94±35		0	
		108±16			0
Histamine	0·1	25±13	20±15		
		18±7		26±23	
		35±4			6±11
	0·5	46±14	57±16		
		41±14		67±22	
		77±40			71±42
	1·0	145±51	91±25		
		103±36		121±23	
		127±31			137±79

responded only slightly, or not at all, to adrenaline, but still retained their sensitivity to histamine. Thus, the mean values for the effect of adrenaline on the contraction (5.0 ± 5 mm.) and on the rate (11.4 ± 12 per min.), after the combined treatment, differed significantly ($P<0.05$) from the corresponding mean values (39.8 ± 6 mm. and 75.8 ± 16 per min.) obtained after treatment with reserpine alone. Dichloroisoproterenol (1 μg./ml.) did not reduce the effect of histamine on the contraction in the same auricles, but appeared slightly to depress its stimulant effect on the rate.

Effects of Histamine Before and After Diphenhydramine and Pempidine on Isolated Guinea-pig Auricles Previously Treated with Reserpine (Figs. 3 and 4).—When ganglionic-blocking agents (hexamethonium 100 μg./ml.; pempidine 0.1 μg./ml.) were allowed to act for 30 min. on auricles which had been previously treated with reserpine, the response to histamine was not affected. When they were allowed to act for 90 min., the stimulant action of histamine on the auricles was no longer obtained. Diphenhydramine (5 μg./ml.), applied to auricles which had been previously treated with reserpine (5 μg./ml. for 5 hr.), reduced or abolished the stimulant effect of histamine on the rate and force of contraction, but did not affect the response of the auricles to adrenaline.

Effects of Histamine, Adrenaline, Noradrenaline, and Nicotine on the Isolated Heart of the Guinea-pig, Before and After Treatment with Dichloroisoproterenol (Table III, Fig. 5).—Dichloroisoproterenol acting in a concentration of 1 μg./ml. was without effect on the heart. Concentrations of 5 to 10 μg./ml. reduced the contraction to $59.8\% \pm 11$, and the rate to $95.3\% \pm 4$, of the original values.

The stimulant effects of adrenaline and noradrenaline and the diphasic effect of nicotine all disappeared in hearts treated with dichloroisoproterenol in concentrations of 1 to 10 μg./ml. Within this range of concentrations, dichloroisoproterenol did not depress the response to small doses of histamine.

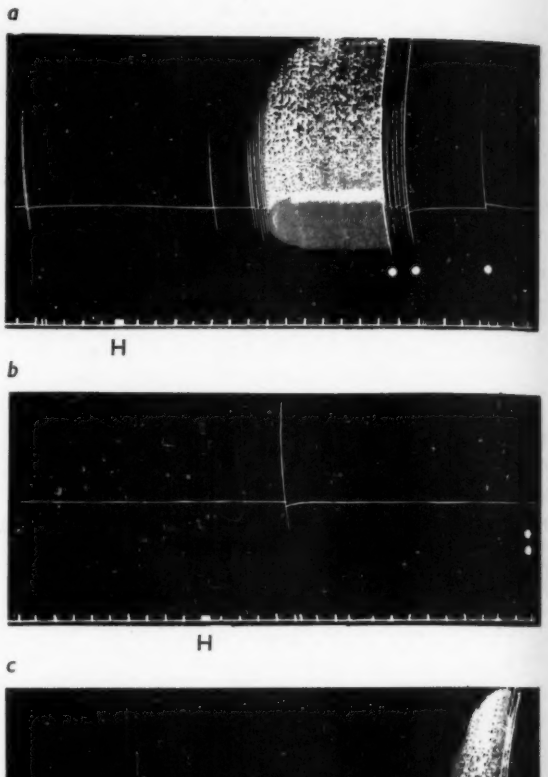

FIG. 4.—Diphenhydramine 5×10^{-6} prevents the response to histamine of guinea-pig auricles previously treated with reserpine. (*a*) Response to histamine 1×10^{-6} (*H*) on guinea-pig auricles previously treated with reserpine 5×10^{-6}. (*b*) Histamine 1×10^{-6} (*H*) 1 hr. after treatment with diphenhydramine 5×10^{-6}. (*c*) Adrenaline 2×10^{-7} (*A*) 90 min. after the treatment with diphenhydramine 5×10^{-6}. Upper tracing: height of contraction. Lower tracing: time, 20 sec. The washings are indicated by the white dots.

DISCUSSION

The results obtained in the present experiments suggest that histamine stimulates isolated cardiac tissue directly, and not through the release of catecholamines. They show that the stimulant action of histamine persisted in auricles which were first treated with reserpine, and then rendered insensitive to adrenaline and noradrenaline by suitable doses of dichloroisoproterenol. Went *et al.* (1952) reported that ergotamine abolished the stimulant effect of histamine on the isolated mammalian heart, and

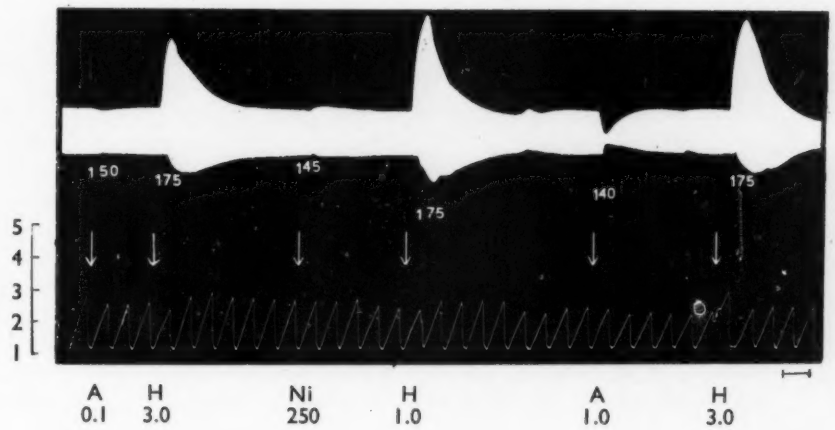

FIG. 5.—Dichloroisoproterenol 1×10^{-6} inhibits the effect of the adrenaline (*A*) and nicotine (*Ni*) in isolated guinea-pig heart, while histamine (*H*) stimulation is still present. Upper tracing: height of contraction. Lower tracing: flow of the perfusion fluid. The numbers indicate heart rate per min. Time, 2 min.

took this as indirect evidence for the release of adrenaline. However, not only is the anti-adrenaline action of ergotamine and related substances on the isolated mammalian heart open to some doubt (Nickerson, 1949), but the results of the present work fail to support the conclusion that histamine acts through the release of adrenaline.

It is unlikely that histamine exerted an effect on ganglia since its action was unaffected by ganglionic blocking agents which were applied in concentrations that abolished the diphasic action of nicotine. The action of ganglionic blocking drugs in abolishing the effect of histamine, after prolonged contact with the preparations, has been discussed elsewhere (Pepeu, Mannaioni, and Giotti, 1958, 1959). The fact that anti-histaminic drugs antagonized the stimulant effect of histamine without modifying the action of adrenaline and noradrenaline may be taken as further evidence for the existence of specific receptors for histamine in cardiac muscle. These findings, however, do not exclude the possibility that histamine acts through the release of stimulating substances which are not antagonized by dichloroisoproterenol. Went *et al.* (1954) have reported that when isolated perfused hearts are stimulated with histamine, substances which inhibit plain muscle appear in the perfusate. These substances have not yet been identified. The appearance of catecholamines in the perfusate during stimulation by histamine would suggest, in the light of the present findings, that their formation or release was not the cause, but the result, of the direct stimulant action of histamine on the heart.

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NEUROMUSCULAR BLOCKADE BY STREPTOMYCIN AND DIHYDROSTREPTOMYCIN

BY

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Both streptomycin and dihydrostreptomycin in large doses produced neuromuscular blockade in different types of skeletal nerve-muscle preparations. Streptomycin had a quicker, more marked and a longer-lasting action than dihydrostreptomycin. Neostigmine reversed a partial block of streptomycin, but a complete neuromuscular block remained unaffected. Calcium ions antagonized the neuromuscular block of streptomycin rapidly but incompletely.

Both streptomycin and dihydrostreptomycin may cause a number of untoward phenomena, particularly injury to the nervous system and hypersensitivity reactions. The main toxic side-effect in the chronic use of streptomycin is damage to the vestibular mechanisms and the eighth cranial nerve, which was first described in man by Hinshaw and Feldman (1945) and in experimental animals by Molitor and his collaborators (1946, 1950). The latter authors also showed that streptomycin in high dosage caused death in experimental animals as a result of respiratory failure. Brazil and Corrado (1957) studied the motor effects of streptomycin in dogs and in pigeons, and they found that streptomycin possesses neuromuscular blocking properties which are similar to those produced by magnesium. Calcium and neostigmine suppressed the motor effects of acute streptomycin intoxication. Brazil and Corrado (1957) employed a very high dose (110 mg./kg.) to demonstrate the neuromuscular blocking effect of streptomycin in dogs, and the effect was not compared with that of dihydrostreptomycin, which is known to differ from streptomycin in toxicity in certain respects. Recently Loder and Walker (1959) have reported 3 cases of muscular paralysis in patients who were kept on streptomycin treatment for a period varying from 3 days to 3 months with usual dosage. It was therefore thought necessary to investigate the pharmacological actions of streptomycin on various types of skeletal muscles and compare its effects with those of dihydrostreptomycin. The results of such a study are reported in this paper.

METHODS

Streptomycin and dihydrostreptomycin were purchased locally of "Hindustan Antibiotics" make; the purity was stated by the manufacturers to conform to the standards prescribed by the *British Pharmacopoeia*.

Rat Phrenic Nerve-Diaphragm Preparation.—This was prepared as described by Bülbring (1946). Ten adult rats were used. The isolated tissues were suspended in a 60 ml. bath, containing Tyrode solution with double the amount of dextrose. Single shocks at rates not exceeding 8/min. were applied to the nerve from a square wave stimulator, at 10 to 12 volts and 0.5 to 1.0 msec. duration. Drugs were added directly to the bath through a very fine polythene tube attached to a hypodermic needle.

Dog Sciatic-Gastrocnemius Preparation.—Experiments were performed in 10 dogs anaesthetized with sodium pentobarbitone (35 mg./kg.) injected intraperitoneally. One leg was immobilized and the sciatic nerve was exposed between the hamstring muscles and sectioned. The peripheral stump of this nerve was placed on a non-polarizing bipolar electrode. The contractions of the gastrocnemius muscle were recorded with an isometric lever. The muscle was stimulated through the sciatic nerve by a square wave stimulator at a rate of 8/min. at 10 to 12 volts and 0.5 to 1.0 msec. duration. Retrograde injections of drugs were made through the cannulated common iliac artery.

Frog Rectus Abdominis Muscle Preparation.—Frogs were pithed and the rectus abdominis muscle was dissected and suspended in a 10 ml. bath containing oxygenated frog-Ringer solution at room temperature. The contractions produced by acetylcholine chloride (0.10 to 1.0 μg./ml.), when added to the bath and left in contact with the tissue for 90 sec., were recorded. Streptomycin and dihydrostreptomycin were added to the bath 30 sec. before a subsequent addition of acetylcholine and the contractions again recorded for 90 sec. The bath was then washed out. Between successive additions of acetylcholine there was at least an interval of 8 min., or until the muscle recovered from the effect of the streptomycins.

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Results

Effect on Rat Diaphragm Preparations. — Streptomycin and dihydrostreptomycin were tested in concentrations ranging from 10 μg. to 1.5 mg./ml. Neither substance affected the contractions in concentrations up to 200 μg./ml. However, at 0.25 mg./ml. streptomycin sulphate produced a mean reduction of 44%, and dihydrostreptomycin at the same dose produced a mean reduction of 19%. Higher concentrations produced proportionately greater effects, a maximum of 94% reduction with streptomycin 1.5 mg./ml., and of 71% with the same dose of dihydrostreptomycin (Table I). It was observed that there was a short latent period in the onset of action with dihydrostreptomycin which was not seen with streptomycin. Recovery of the diaphragmatic contractions was complete after washing out the drug, the recovery time varying according to the dose. Higher concentrations took a longer time for the complete recovery of the muscle. Muscle treated with dihydrostreptomycin took a shorter time for recovery in comparison with streptomycin. The response to direct excitation of the rat diaphragm did not alter in any of the experiments.

TABLE I

THE MEAN REDUCTION IN MUSCULAR CONTRACTIONS PRODUCED BY STREPTOMYCIN AND DIHYDROSTREPTOMYCIN IN THREE DIFFERENT PREPARATIONS

Muscle Preparation	Dose	% Reduction in Contraction	
		Streptomycin	Dihydrostreptomycin
Rat phrenic nerve-diaphragm	mg./ml. 0·25	44	19
	0·5	57	52
	1·0	94	71
Dog gastrocnemius-sciatic nerve	mg./kg. 10	20	13
	20	83	16
	40	100	24
Frog rectus abdominis	mg./ml. 5	37	11
	10	40	27
	20	46	38

Effect on Dog Gastrocnemius Sciatic Preparation. — Streptomycin and dihydrostreptomycin were injected intravenously in doses ranging from 1 mg. to 40 mg./kg. body weight. Doses below 8 mg./kg. did not affect the contractions of the gastrocnemius, whereas higher doses produced graded reductions in the amplitude of the contractions (Table I and Fig. 1*a*). The effect of dihydrostreptomycin was not only slower to develop but the overall depression in muscular contraction was much less (Table I and Fig. 1*b*). As in the rat diaphragm, recovery was quicker with dihydrostreptomycin.

Neostigmine given intravenously in 1.5 mg. doses antagonized the effect of a partial neuromuscular block of streptomycin (Fig. 2*a*), but in the presence of total neuromuscular block neostigmine failed to restore the contractions (Fig. 2*b*). Calcium chloride, 0.5 mg./kg., on the other hand, antagonized the neuromuscular block of streptomycin quickly, but not completely (Fig. 2*b*).

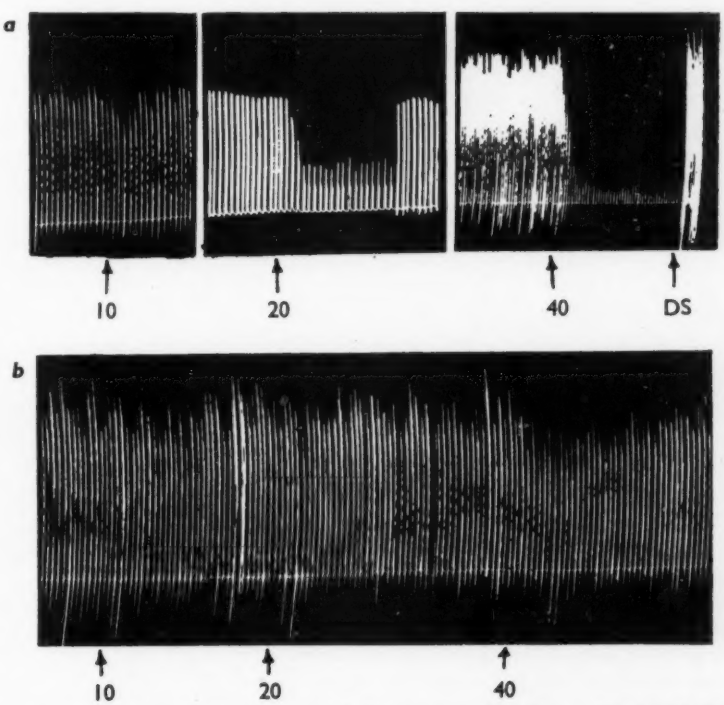

FIG. 1.—Dog gastrocnemius-sciatic nerve preparation. Contractions induced by indirect stimulation of the sciatic nerve or by direct stimulation of the muscle. In (*a*) effect of streptomycin 10, 20, and 40 mg./kg. and of direct stimulation (DS). In (*b*) effect of dihydrostreptomycin 10, 20, and 40 mg./kg.

FIG. 2.—Dog gastrocnemius-sciatic nerve preparation. Contractions induced by indirect stimulation of the sciatic nerve or by direct stimulation of the muscle. Effect of streptomycin and antagonists. At S20, 20 mg./kg. streptomycin. NS, neostigmine 0.15 mg./kg. S40, 40 mg./kg. streptomycin. DS, direct stimulation. T, time of recovery—30 min. after first injection of streptomycin. T_1, time of recovery—25 min. after second injection of streptomycin. Ca, calcium chloride 10%—0.5 ml./kg.

Effect on Frog Rectus-Abdominis Muscle Preparation.—The effects of streptomycin and dihydrostreptomycin were tested on the acetylcholine-induced contractions of the rectus abdominis muscle. Graded doses of streptomycin and dihydrostreptomycin were given ranging from 1 mg. to 20 mg./ml. Doses from 5 to 20 mg./ml. inhibited the contractions of the rectus muscle to a varying degree, and the effect was proportional to the concentration of drug in the bath. Streptomycin had a greater effect than dihydrostreptomycin; 20 mg./ml. of streptomycin depressed the muscle so as to prevent its recovery for as long as 2 hr. even after repeated washings of the muscle. In contrast to this, recovery of the muscle was complete in the case of dihydrostreptomycin after a period of 1 hr. (Fig. 3*a* and *b*).

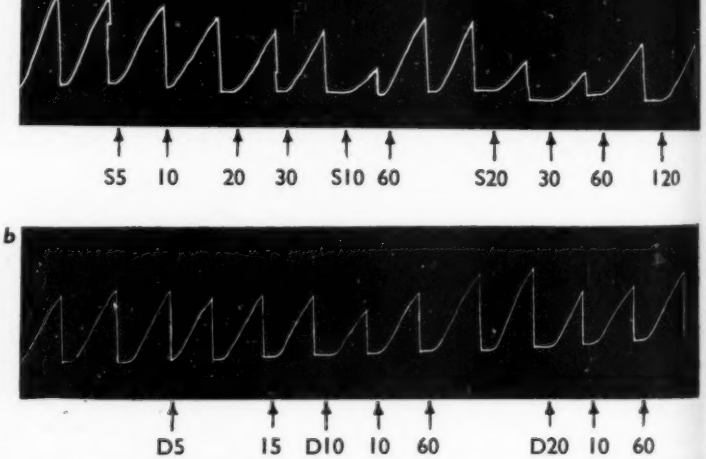

FIG. 3.—Frog rectus abdominis suspended in a 10 ml. bath containing oxygenated frog-Ringer solution at room temperature. Contractions to acetylcholine (1.0 μg./ml.). In (*a*) effects of streptomycin and in (*b*) effects of dihydrostreptomycin. Numbers indicate time intervals in min. Acetylcholine was left in the bath for 90 sec. and then washed out. At S5, 5; S10, 10; and S20, 20 mg./ml. streptomycin. At D5, 5; D10, 10; and D20, 20 mg./ml. dihydrostreptomycin.

DISCUSSION

It has been clearly demonstrated that streptomycin can cause muscular paralysis in high doses, thus confirming the observations of Brazil and Corrado (1957). The muscular paralysis is much more marked with streptomycin than dihydrostreptomycin when equal doses are used. The onset of action is more rapid with streptomycin and the recovery time of muscle is definitely shorter with dihydrostreptomycin. The muscular paralysis seems to be due to neuromuscular blockade produced by streptomycin, since the direct excitability of the different muscle preparations was never found to be decreased in any of the experiments. Neuromuscular blockade was demonstrated in three different types of skeletal muscle preparations, which included mammalian as well as amphibian skeletal muscles. This lends support to the view that all types of muscles are susceptible to the neuromuscular blocking action of streptomycin in high doses. In contrast to Brazil and Corrado (1957), who studied only one type of skeletal muscle preparation (the sciatic-tibialis anticus of the dog) and used a high dose (110 mg./kg.), in these experiments we found that a dose of 40 mg./kg. produced complete paralysis and smaller doses had proportionately a smaller effect. It is therefore not unlikely that streptomycin in ordinary therapeutic doses may be capable of producing neuromuscular blockade in certain isolated cases, as has been observed by Loder and Walker (1959). The neuromuscular blockade with streptomycin can possibly occur as

a result of cumulative action of the drug which is usually administered over a prolonged period.

Neostigmine antagonizes the effect of streptomycin only when there is a partial neuromuscular block. In the presence of complete neuromuscular block the effect of neostigmine is negligible. Calcium ions can antagonize quickly but less completely the effect of streptomycin on skeletal muscles. Similar results have been reported by Corrado, Ramos, and De Escobar (1959) for the neuromuscular blocking action of neomycin. It is thus likely that the neuromuscular blocking actions of neomycin and streptomycin involve a common mechanism of action.

Another interesting feature of the present investigation is the difference in action of streptomycin and dihydrostreptomycin. Clinically streptomycin differs from dihydrostreptomycin regarding the injury it is likely to cause to the nervous system in general and damage to the eighth cranial nerve in particular. We have found that neuromuscular blockade produced by dihydrostreptomycin is not only slow to develop, but the depression of muscular contraction is less and the time for recovery of the muscles is quicker, as compared with streptomycin.

The present experiments have thus not only confirmed the clinical case reports of Loder and Walker (1959), but also suggest that the danger of neuromuscular blocking action of streptomycin would be greatly diminished by using dihydrostreptomycin. On the other hand, dihydrostreptomycin may cause a greater incidence of auditory damage than streptomycin (Crofton, 1960). The antagonists to the toxicity on skeletal muscles resulting from the use of streptomycin, as suggested from these observations, should be both neostigmine and calcium. Simultaneous administration of calcium throughout streptomycin therapy is suggested as a prophylactic measure against the toxicity of streptomycin on skeletal muscles.

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THE EFFECT OF LEVALLORPHAN TARTRATE AND OF ADIPHENINE HYDROCHLORIDE ON THE ANTIDIURETIC ACTION OF MORPHINE AND NICOTINE

BY

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Levallorphan antagonizes the antidiuretic effect of morphine. It is at least 25 times as potent as nalorphine in this respect. However, neither levallorphan nor adiphenine antagonized the antidiuretic action of nicotine.

Morphine and nicotine are both known to inhibit the diuretic response to water, an action mediated through the hypothalamic neurohypophysial system (De Bodo, 1944; Burn, Truelove, and Burn, 1945; Bisset and Walker, 1957). Nalorphine has been shown to antagonize the antidiuretic effect of one of these compounds—namely, morphine (Winter, Gaffney, and Flataker, 1954; Schnieden and Blackmore, 1955) and levallorphan is known to be a very potent antagonist of certain of the actions of morphine (Fromherz and Pellmont, 1952). It therefore seemed of interest to see if this latter compound antagonized the antidiuretic effects of both morphine and nicotine. In addition, adiphenine hydrochloride ("Trasentin") has also been reported to have certain antinicotinic actions (Tripod, 1949), and this compound was therefore also tested to see if it would antagonize the antidiuretic effects of nicotine.

METHODS

Adult albino rats weighing approximately 250 g. were used. The effect of drugs on the diuretic response to water was tested by the method of Schnieden and Blackmore (1955) on groups of 16 animals. Initially the animals in the group were randomly allocated to one of the drug treatments, and cross-over experiments were then carried out at weekly intervals until all 16 animals in the group had received all treatments. All drugs except adiphenine in a dose of 31 mg./kg. (Table II) were given subcutaneously 45 min. after the second dose of water was administered. The water load remaining at this time was considered the initial water load. For the next 90 min. urine volumes were noted at 30 min. intervals. The following drugs were injected: nicotine hydrogen tartrate 7.5 mg./kg., morphine sulphate 10 mg./kg., nalorphine hydrobromide 25 mg./kg., levallorphan tartrate 0.1 or 1 mg./kg., adiphenine hydrochloride 21 or 31 mg./kg. Control groups received corresponding injections of 0.9% sodium chloride solution. In all instances except one (Table II) the antidiuretic drug and its possible antagonist were injected simultaneously into different subcutaneous sites. Analgesic effect in rats was estimated by the time required from exposure to a standard thermal stimulus until the rat moved its tail (Davies, Raventós, and Walpole, 1946). To avoid injury to the animal's tail exposure was limited to 15 sec.; failure of the animals to react in this time was considered to indicate "complete analgesia."

RESULTS

Levallorphan and nalorphine are capable of antagonizing the inhibition of the renal response to a water load produced by morphine as illustrated in Table I. This Table also shows that 1 mg./kg. levallorphan produces approximately the same degree of antagonism as 25 mg./kg. nalorphine. Neither levallorphan nor nalorphine had an antidiuretic effect in rats in the doses used; when comparing the effect of 0.9% sodium chloride solution with nalorphine, $t = 1.7$ $P<0.1>0.05$.

Neither adiphenine nor levallorphan antagonized the antidiuretic effect of nicotine, and adiphenine had no significant effect on the renal response to a water load (Table II); when comparing the effect of 0.9% sodium chloride solution with adiphenine, $t = 1.9$ $P<0.1>0.05$. However, adiphenine in a dose of 21 mg./kg. enhanced the antidiuretic effect of nicotine; when comparing nicotine with nicotine plus adiphenine, $t = 2.4$ $P<0.05>0.01$.

TABLE I

THE EFFECT OF NALORPHINE AND LEVALLORPHAN ON THE ANTIDIURETIC ACTION OF MORPHINE

Figures represent percentage of initial water load excreted during the 90 min. period after injection of the drugs (mean±S.E. for 16 animals)

Saline	Nalorphine	Levallorphan	Morphine	Morphine/ Nalorphine	Morphine/ Levallorphan	Dose (per kg. Body Wt.) Levallorphan
91·7± 9·3	73·3±5·4	—	5·3±2·1	69·7±6·2	—	—
123·7±17·6	—	109·5±13·2	18·0±2·9	—	48·6±17·6	0·1 mg.
109·2±11·2	—	107·6±19·2	4·8±1·2	—	65·0±10·6	1·0 ,,

TABLE II

THE EFFECT OF LEVALLORPHAN AND ADIPHENINE ON THE ANTIDIURETIC ACTION OF NICOTINE

Figures represent percentage of initial water load excreted during the 90 min. period after injection of the drugs (mean±S.E. for 16 animals). Adiphenine 31 mg. was given 45 min. before nicotine.

Saline	Nicotine	Nicotine/ Adiphenine	Nicotine/ Levallorphan	Adiphenine	Dose (per kg. Body Wt.)
88·6±8·6	34·7±6·4	—	41·9±7·5	—	Levallorphan 1 mg.
67·4±4·9	19·3±3·4	8·7±2·4	—	56·2±3·2	Adiphenine 21 ,,
70·8±8·6	24·3±9·8	24·4±8·7	—	57·4±9·2	,, 31 ,,

TABLE III

EFFECT OF LEVALLORPHAN ON MORPHINE ANALGESIA

The number of animals which showed "complete analgesia" (>15 sec.) to a standard thermal stimulus.

Drug	No. of Animals Tested	Time after Injection	
		40 min.	70 min.
Morphine 10 mg./kg.	16	13	12
Morphine 10 mg./kg./ levallorphan 0·1 mg./kg.	16	10	11
Morphine 10 mg./kg.	8	8	8
Morphine 10 mg./kg./ levallorphan 1 mg./ kg.	8	0	0

Increasing the dose of levallorphan not only antagonized the antidiuresis of the morphine-treated animals but also decreased the analgesic effect of morphine. Levallorphan 0.1 mg./kg. had no appreciable effect in antagonizing the analgesic action of morphine; in doses of 1 mg./kg., however, its effect was more pronounced (Table III).

DISCUSSION

Morphine and nicotine both stimulate neurohypophysial secretion (De Bodo, 1944; Giarman, Mattie, and Stephenson, 1953; Duke, Pickford, and Watt, 1951; Bisset and Walker, 1957) presumably by hypothalamic stimulation. It appears likely from the work of Pickford (1939, 1947, 1953) that the pathway for the antidiuretic response involves a synapse at the supraoptic nucleus. Bisset and Walker (1957) have suggested that such synapses differ from synapses at autonomic ganglia. They noted that hexamethonium in doses sufficient to block the pressor and convulsant actions of nicotine had no effect on its antidiuretic effect. Adiphenine has been shown by Tripod (1949) to have marked antinicotinic and anti-acetylcholine effects. Like hexamethonium, it can also inhibit the convulsant actions of nicotine (Tripod, 1949) but not its antidiuretic effects.

Pickford (1939) noted that acetylcholine injected intravenously inhibited a water diuresis but that

pretreatment with atropine did not prevent this inhibition. More recently Supek and Eisen (1953) have shown that the antidiuretic effect of nicotine also is not blocked by atropine. Since adiphenine has an atropine-like action the present findings are consistent with those of Supek and Eisen (1953). These workers also noted that other atropine-like compounds and ganglionic blocking compounds such as pentamethonium, parpanit, and diparcol were also without effect.

An injection of morphine into the supraoptic nucleus has been shown to inhibit water diuresis. Since levallorphan is a competitive antagonist of morphine it presumably acts by competing with it for tissue receptors. Beckett, Casy, Harper, and Phillips (1956) are of the opinion that the stereochemical structure of these antagonists are important in determining the goodness of fit of such compounds for the analgesic receptor site. It is likely that nicotine acts in a different way from morphine, since Pickford (1953) showed that the injection of dyflos into the supraoptic nucleus abolished the antidiuretic action of acetylcholine (which presumably has an action similar to nicotine) but not that of morphine—hence the inability of levallorphan to antagonize nicotine might be expected.

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THE EFFECT OF DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE SMALL INTESTINE AND OTHER ORGANS IN RATS AND MICE

BY

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In rats, a diet of meat or liver caused a rise in the amount of 5-hydroxytryptamine in the small intestine, an increase in the weight of the intestine and a 10-fold increase in anaerobic bacteria in the rectum. A diet containing added tryptophan did not affect tissue levels of 5-hydroxytryptamine in rats, but in mice there was a rise of 5-hydroxytryptamine in various tissues. The significance of these findings is discussed together with the possible role of intestinal bacteria in mediating the effects caused by a meat diet. No change was observed in the 5-hydroxytryptamine content of brain or in the distribution of argentaffin cells in the jejunum as a result of these diets.

Cordier (1926) reported that the argentaffin cells in the intestine of mice were increased in number 5 to 10 times and showed increased granularity when the animals received a diet exclusively of raw meat for 7 to 10 weeks. Since argentaffin cells are believed to secrete 5-hydroxytryptamine (Erspamer, 1954 ; Benditt and Wong, 1957), Stacey and Sullivan (1957) studied the effect of a diet of raw meat on the 5-hydroxytryptamine content of the small intestine of rats, and reported that it was raised. They also found that the 5-hydroxytryptamine content of the small intestine in mice was raised when tryptophan was added to the diet, and concluded that the high tryptophan intake in an exclusively meat diet might account for the rise of intestinal 5-hydroxytryptamine. The present paper reports further studies on the effect of protein feeding on 5-hydroxytryptamine metabolism in rats and mice.

METHODS

Groups of adult male rats weighing 120 to 200 g. or mice weighing 20 to 25 g. were matched according to age and weight. Controls received a mixed balanced diet supplied by J. Murray and Sons, London, S.E.8, in cube or powder form, and treated groups received the experimental diet. Both groups were given unrestricted water and a vitamin supplement (Abidec drops ; Parke, Davis and Co.) in their drinking water twice weekly. Both groups remained healthy, although differences in weight between control and treated groups sometimes developed during treatment.

Aliquots of a homogenate of the whole small intestine from the pyloric sphincter to the ileo-caecal junction of mice were assayed. Since the whole rat small intestine is bulky, tough and difficult to homogenize completely, the mucous membrane, which contains all the 5-hydroxytryptamine (Feldberg and Toh, 1953), was scraped off with a scalpel and homogenized ; aliquots were then assayed.

The 5-hydroxytryptamine content of the small intestine and spleen was usually assayed fluorimetrically by the methods of Udenfriend, Weissbach and Clark (1955) or Weissbach, Waalkes, and Udenfriend (1958). Sometimes, as indicated in the text, 5-hydroxytryptamine was assayed biologically. Fluorescence was measure in 3N hydrochloric acid, in which tryptamine and tryptophan do not fluoresce (Udenfriend, Bogdanski, and Weissbach, 1955). For bioassay, 5-hydroxytryptamine was extracted with 95% acetone and assayed on the atropinized oestrous rat uterus by the superfusion technique (Gaddum, 1953). Brain 5-hydroxytryptamine was assayed by the method of Bogdanski, Pletscher, Brodie, and Udenfriend (1956).

Since the treated animals lost weight during the experiment, results expressed on the basis of tissue weight or final body weight are fallacious. However, as body weights at the beginning of the experiment were similar for the two groups, the concentrations of 5-hydroxytryptamine are expressed in μg./kg. initial body weight except where stated otherwise.

Some variation will be seen between the results obtained for control groups because of differences between them in the initial body weight. There was closer agreement when the results were expressed as μg./g. tissue.

For estimations of the 5-hydroxytryptamine content of platelets, rats were lightly anaesthetized with ether, and bled from the carotid artery either through a piece of polythene tubing not more than 2.5 cm. long or a siliconed glass cannula. Mice were decapitated after light ether anaesthesia and the blood collected via a siliconed glass funnel into a siliconed glass tube containing 1 ml. of disodium edetate solution (1.0 g./100 ml. 0.7% sodium chloride). The volume of blood obtained was about 1 ml. from mice and 6 ml. from rats. Platelet-rich plasma was prepared by the method of Hardisty and Stacey (1955), except that the blood was centrifuged for 10 min. at 160 *g*. The relatively high proportion of sodium edetate present did not interfere with the fluorimetric estimation since it neither fluoresced nor caused quenching of the fluorescence due to 5-hydroxytryptamine.

Urinary excretion of 5-hydroxyindoleacetic acid was estimated on the pooled urine of groups of rats by the method of Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus, and Smith (1956) after collecting urine from the rats in metabolism cages for 16 to 18 hr. overnight. For estimation of the percentage recovery of injected 5-hydroxytryptamine as 5-hydroxyindoleacetic acid, rats were injected subcutaneously with 6 mg./kg. 5-hydroxytryptamine as the creatinine sulphate after which urine was collected for 16 to 18 hr. overnight. Controls received 0.5 ml. saline subcutaneously. Urinary creatinine was estimated by the microchemical method of Folin (1914).

For observation of the rate at which 5-hydroxytryptamine was lost from the small intestine, consecutive pieces of jejunum starting from the duodenal junction, each 1.8 to 2.5 cm. long, were incubated for 0.5, 1.0, 1.5, and 2.0 hr. in Krebs Ringer phosphate solution at 37° with gentle shaking. The pieces were then washed in fresh, ice-cold Krebs solution and assayed for 5-hydroxytryptamine.

In some experiments rectal swabs were taken from animals on the day they were killed. They were plated on blood agar and MacConkey's media. The former plates were incubated aerobically and the MacConkey's plates anaerobically in a McIntosh and Fildes' jar. After 24 hr. the colonies were counted.

Sections of small intestine taken for histology of the argentaffin cells were fixed in Bouin's fluid, or in 10% formol, and embedded in paraffin. The sections were stained with Masson's silver stain or by the activated Protargol method (Schofield, 1951).

Results

Experiments with Meat Diet

Rats.—Rats were fed on a diet of raw lean beef for either 10 to 23 days or 60 to 67 days before 5-hydroxytryptamine was assayed fluorimetrically. Table I shows the results of these experiments. There was a significant rise of intestinal 5-hydroxytryptamine in the meat-fed rats after both periods of treatment. The 5-hydroxytryptamine content of spleen, platelets and brain was not affected after 10 to 23 days of treatment, however.

These results were confirmed when the intestinal 5-hydroxytryptamine content of groups of rats was assayed biologically after 20, 43 and 76 days on a meat diet. At the end of each of these periods of treatment the 5-hydroxytryptamine level was higher in treated than in control rats. The overall mean rise in 7 treated rats was 102%, but since the numbers of animals in the 3 experiments were not strictly comparable, the overall results have not been statistically analysed.

Urinary excretion of 5-hydroxyindoleacetic acid was estimated at various times during these

TABLE I

THE EFFECT OF A MEAT DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE TISSUES OF RATS IN 2 EXPERIMENTS OF DIFFERENT DURATION

Mean results are expressed as μg. 5-hydroxytryptamine/kg. body weight ± standard error, except for platelets, where results are expressed as μg. 5-hydroxytryptamine/ml. blood. n.s.=difference not significant ($P>0.05$).

Length of Treatment (days)	No. of Rats		5-Hydroxytryptamine Content			Tryptophan Intake mg./kg./day	
10–23	Controls	Treated	Controls	Treated	Significance	Controls	Treated
Small intestine	16	16	67·0 ±3·65	89·0 ±3·78	$P<0.001$		
Brain	10	10	6·0 ±0·55	5·1 ±0·37	n.s.		
Spleen	16	15	26·3 ±3·26	27·4 ±5·3	n.s.	116	808
Platelets	4	5	1·42±0·09	1·67±0·14	n.s.		
60–67							
Small intestine	5	5	106·0 ±8·68	157·0 ±14·9	$P<0.02$		

experiments. Four estimations were carried out during the first 10 days of treatment and showed no difference between the groups; the mean excretion for 6 control rats was 234 ± 30 μg./kg./24 hr., and for 6 meat-fed rats 239 ± 26 μg./kg./24 hr. Since it was possible that there might be a time lag before changes in 5-hydroxyindoleacetic acid excretion occurred, estimations were carried out after further periods of meat feeding. The results are shown in Table II. Although the excretion of 5-hydroxyindoleacetic acid by meat-fed rats was lower throughout, excretion by both groups varied considerably. Excretion of creatinine, though lower in meat-fed rats, was less variable.

TABLE II

URINARY EXCRETION OF 5-HYDROXYINDOLEACETIC ACID AND CREATININE BY 6 CONTROL AND 6 MEAT-FED RATS AT VARIOUS TIMES DURING TREATMENT

Duration of Treatment (days)	Group	5-Hydroxyindoleacetic Acid μg./kg./24 hr.	Creatinine mg./kg./24 hr.	5-Hydroxyindoleacetic Acid/Creatinine Ratio
11	Control	334	34	10·0
	Meat-fed	283	28	10·1
17	Control	367	29	12·9
	Meat-fed	247	21	12·1
51	Control	214	29	7·5
	Meat-fed	166	26	6·5
80	Control	187	30	6·2
	Meat-fed	134	26	5·3
169	Control	166	32	5·2
	Meat-fed	122	29	4·2

To investigate whether control and meat-fed rats metabolized injected 5-hydroxytryptamine differently, the percentage recovery of injected 5-hydroxytryptamine as urinary 5-hydroxyindoleacetic acid was studied. From 9 control rats the recovery was 28.5%, and from 9 rats treated for 20 days 30.5%. Erspamer (1955) gave similar figures for the recovery of injected 5-hydroxytryptamine.

In all these experiments it was found that the intestinal mucous membrane of rats which were meat-fed weighed more than in controls. The figures are shown in Table III. In the first experiment, lasting 10 to 23 days, the difference was significant. When the mucosal and muscle layers of the intestine were weighed separately it was found that in both control and treated groups the mucosal layer formed approximately the same proportion of the total weight of the intestine, namely 25 to 30%, indicating that the weight increase involved both mucosal and muscle layers.

This phenomenon was not a reflection of differences in body weight between control and treated groups. As shown in Table III, the mean body weights of the groups were very close. In the second experiment, lasting 60 to 67 days, the final body weight of the meat-fed rats was a little lower than that of controls, although their intestinal mucosa was heavier.

The weight increase of the intestine did not account for the rise in 5-hydroxytryptamine content, because the mucosa of meat-fed rats

TABLE III

THE EFFECT OF A MEAT DIET ON THE WEIGHT AND 5-HYDROXYTRYPTAMINE CONTENT OF THE SMALL INTESTINAL MUCOSA AND THE BODY WEIGHT OF RATS

All results given as means ± standard error. n.s.=difference not significant ($P>0.05$).

Duration of Treatment (days)	Group	No. of Rats	Wt. of Intestinal Mucosa (g.)	5-Hydroxytryptamine μg./g. Mucosa	Body Weight (g.)	
					Initial	Final
10 to 23	Control	16	3·84±0·16	3·30±0·14	188±5·8	210±7·6
	Meat-fed	15	4·52±0·13	3·81±0·16	194±6·9	215±6·2
			$P<0.01$	$P<0.05$		
60 to 67	Control	5	3·53±0·21	3·57±0·19	120±2·3	241±0·6
	Meat-fed	5	4·14±0·30	4·93±0·18	131±3·7	231±3·5
			n.s.	$P<0.001$		

contained more 5-hydroxytryptamine per g. of tissue than did control mucosa (Table III).

Since it was thought possible that alterations of intestinal bacterial flora due to meat-feeding might contribute towards the rise in weight of the intestine or in its 5-hydroxytryptamine content, rectal swabs were taken from 9 control and 9 meat-fed rats. The growth of aerobes was qualitatively and quantitatively similar in treated and control groups, but the anaerobic growth was much more profuse in meat-fed rats. The ratio of the number of anaerobic colonies after 24 hr. incubation between meat-fed and control rats was of the order of 10:1. Qualitative differences were not investigated.

In view of the histological findings in meat-fed mice reported by Cordier (1926), sections of jejunum from control and treated rats were stained for argentaffin cells. In control specimens under the oil immersion (magnification ×1,000) 425 argentaffin cells were counted in 400 fields, or 1.06 cells/field. In the intestine of meat-fed rats there were 392 argentaffin cells in 420 fields, or 0.94 cells/field. No difference in the number of granules in the argentaffin cells of the two groups was observed. An attempt was made to count the number of granules per cell, but in many cases the intensity of granulation made it impossible to distinguish individual granules. No other histological differences between the intestines of meat-fed and control animals were seen.

Mice.—An attempt was made to repeat the experiment of Cordier on the effect of meat-feeding for 7 to 10 weeks on the argentaffin cells of mice, and to estimate intestinal 5-hydroxytryptamine in addition. By the end of 9 weeks, however, only 2 of the meat-fed mice remained alive. Very few argentaffin cells were seen in stained sections of jejunum. Under the oil immersion (magnification ×1,000) 8 cells were seen in 50 fields in the controls, and 2 in 50 fields in meat-fed mice. The number of surviving mice was too small for any comparison of intestinal 5-hydroxytryptamine content to be made.

Experiments with Tryptophan Feeding

Rats.—By measuring the food intake and using the analytical data of Block and Weiss (1956) it was calculated that the mean dietary tryptophan intake of meat-fed rats was considerably higher than that of the controls (Table I).

If the effect of raising the intestinal 5-hydroxytryptamine of rats as a result of meat-feeding was due to the increased dietary tryptophan intake, it should be possible to reproduce the effect by adding tryptophan to the ordinary diet. Treated rats therefore received the normal diet of rat cubes crushed into powder form to which 1% DL-tryptophan had been added, controls receiving crushed cubes alone.

Three experiments of varying duration were carried out. No change in intestinal 5-hydroxytryptamine was found. Results are shown in Table IV in which rats treated for 22, 28, 40, 60, and 61 days are grouped together for convenience. It will be seen that the tryptophan intake of treated rats was many times greater than that of controls. The 5-hydroxytryptamine content of spleen and brain was also assayed in these animals but no differences were found between treated and controls. No difference in the weight of the intestine was found between the two groups.

Recovery of injected 5-hydroxytryptamine and serial 5-hydroxyindoleacetic acid estimations did not reveal any differences between controls and tryptophan-fed rats.

TABLE IV

THE EFFECT OF 1% TRYPTOPHAN ADDED TO THE CONTROL DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE SMALL INTESTINE OF RATS IN 3 EXPERIMENTS OF DIFFERENT DURATION

Results are expressed as means ± standard error.

Duration of Treatment (days)	No. of Rats		5-Hydroxytryptamine Content μg./kg. Body Weight		Tryptophan Intake mg./kg./day	
	Controls	Treated	Controls	Treated	Controls	Treated
10 to 15	6	5	61·0±6·79	59·0±2·49	116	1,246
22 ,, 61	10	10	64·0±3·43	67·5±5·47	112	832
144 ,, 161	5	6	73·5±0·63	78·0±7·02	109	760

Bacterial growth from rectal swabs was studied. No qualitative or quantitative difference was found between the two groups in aerobic growth. Anaerobic growth from swabs of tryptophan-fed rats was 2.5 times that of the controls.

Mice.—Treated mice received 1% DL-tryptophan in their diet in one experiment for 10 to 12 days and in another for 61 to 63 days. Table V shows the results obtained. In both experiments the 5-hydroxytryptamine content of the small intestine and spleen was significantly raised in the treated mice. Brain 5-hydroxytryptamine was unaffected. Platelet 5-hydroxytryptamine was assayed in the shorter experiment and also found to be raised. The mean weight of the intestine was the same in the 2 groups.

Calculation of the dietary tryptophan intake showed that treated mice had a very much higher intake than controls.

TABLE V

THE EFFECT OF 1% TRYPTOPHAN ADDED TO THE CONTROL DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE TISSUES OF MICE IN 2 EXPERIMENTS OF DIFFERENT DURATION

Mean results are expressed as μg. 5-hydroxytryptamine/kg. body weight ± standard error, except for platelets, where results are expressed as μg. 5-hydroxytryptamine/ml. blood. n.s.=difference not significant ($P>0.05$).

Length of Treatment (days)	No. of Mice		5-Hydroxytryptamine Content			Tryptophan Intake mg./kg./day	
10–12	Controls	Treated	Controls	Treated	Significance	Controls	Treated
Small intestine ..	19	19	172 ±14·1	221 ±14·0	$P<0.05$	230	1,132
Brain	10	10	17·3 ± 0·69	17·3 ± 1·36	n.s.		
Spleen	11	11	59 ± 9·42	108 ±10·6	$P<0.01$		
Platelets	10	10	3·25± 0·28	4·78± 0·39	$P<0.01$		
61–63							
Small intestine ..	8	7	206 ±18·8	315 ±35·0	$P<0.01$	248	1,194
Brain	8	6	16·3 ± 1·42	16·5 ± 2·23	n.s.		
Spleen	8	7	100 ±16·1	168 ±19·3	$P<0.02$		

TABLE VI

THE EFFECT OF OTHER HIGH-PROTEIN DIETS ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE TISSUES OF RATS

Mean results are expressed as μg. 5-hydroxytryptamine/kg. body weight ± the standard error. n.s.=difference not significant ($P>0.05$).

Diets of Groups	No. of Rats	Small Intestine	Spleen	Brain	Tryptophan Intake μg./kg./day
Control	8	100 ± 3·64	15·6±1·36	5·7±0·48	126
Liver	8	151 ±13·5	12·4±1·13	5·0±0·25	324
		$P<0.01$	n.s.	n.s.	
Control	5	91·0± 5·05	16·6±2·24	—	112
Soya Bean	5	91·5±12·4	15·9±1·27	—	265
		n.s.	n.s.		
Control	6	61·0± 6·81	41·5±2·22	—	116
Calcium caseinate ..	5	59·0± 6·65	51·5±9·54	—	481
		n.s.	n.s.		

Other Protein-feeding Experiments

Although meat-feeding of rats produced a significant rise of intestinal 5-hydroxytryptamine, this did not occur in tryptophan-fed rats. The reason for this discrepancy was investigated by feeding high-protein diets in various forms to rats.

Diets composed of ox liver, soya bean flour, and calcium caseinate were fed to groups of rats, matched with controls, for 10 to 20 days, and the results of 5-hydroxytryptamine assays are shown in Table VI.

Only the liver diet produced a rise of the 5-hydroxytryptamine content of the small intestine. As with meat-fed rats, the weight of the intestinal mucosa of liver-fed rats was greater than in controls, the mean weights being 4.52 ± 0.09 g. and 4.07 ± 0.19 g. respectively ($P<0.1$).

Calcium caseinate proved to be a very inadequate diet, since the treated rats lost 15.5% of their body weight while controls gained 9%. This was reflected in the relative weights of the intestinal mucosa, the mean weight for controls being 3.44 ± 0.28 g. and of treated rats 2.55 ± 0.17 g. As shown in Table VI, the intestinal 5-hydroxytryptamine content of rats fed with calcium caseinate expressed as μg./kg. body weight differed little from that of controls. The diminution of intestinal size and weight due to inanition did not affect the total 5-hydroxytryptamine content, but there was a higher concentration per g. of mucosa. The mean 5-hydroxytryptamine content of treated rats was 4.11 ± 0.10 μg./g. and of controls 3.06 ± 0.18 μg./g. of mucosa ($P<0.05$).

No differences between control and treated rats were found in the 5-hydroxytryptamine content of spleen or brain. On all these diets, the tryptophan intake of the treated rats was appreciably higher than that of their controls.

Gut Incubation Experiments

To investigate whether there was any difference between treated and control animals in the rate at which the small intestine gave up 5-hydroxytryptamine into the surrounding medium, pieces of jejunum were incubated as described in "Methods." Experiments were performed on intestine from 3 meat-fed rats, 2 tryptophan-fed rats and 2 tryptophan-fed mice, all of which were matched with controls. In all experiments the rate of loss was about the same for treated and control animals.

Discussion

The results show that a meat diet caused a rise of intestinal 5-hydroxytryptamine in rats and that a liver diet had a similar effect. Since meat feeding in rats produced no effect on the 5-hydroxytryptamine content of the spleen or platelets or on the urinary excretion of 5-hydroxyindoleacetic acid, it seems likely that the effect of this diet was a local one, increasing the storage of 5-hydroxytryptamine in the intestine. The addition of tryptophan to the diet of rats, however, did not affect the 5-hydroxytryptamine content in any of the tissues examined, nor was it affected by feeding the rats on a diet of soya bean flour or calcium caseinate, both of which provided diets rich in tryptophan. The failure of these tryptophan-rich diets to raise intestinal 5-hydroxytryptamine makes it unlikely that the high tryptophan content of the meat diet was wholly responsible for this change. However, the fact that tryptophan supplied in the form of meat may have been better absorbed or better utilized than when added to the ordinary diet cannot be excluded. In this connexion it may be significant that ox muscle contains 77 μg. pyridoxine/100 g. and ox liver 170 μg./100 g., whereas the diet received by control rats was largely composed of cereals and unlikely to contain more than 40 μg. pyridoxine/100 g. (data from Bicknell and Prescott, 1953).

A large increase in the anaerobic bacterial flora was found in the intestine of meat-fed rats. Mitoma, Weissbach, and Udenfriend (1956) have shown that hydroxylation to 5-hydroxytryptophan is one of the major metabolic pathways for tryptophan in *Chromobacterium violaceum*. Many organisms, including the common intestinal organism *E. coli* (Stephenson, 1949), are capable of metabolizing tryptophan, although the pathways involved have so far been incompletely defined, while conversely certain bacteria have been shown to be capable of synthesizing tryptophan (Clifton, 1957). Possibly, therefore, in the altered state of the intestinal bacterial flora in meat-fed rats, bacterial activity might result in both increased formation and hydroxylation of tryptophan.

Another possible mechanism by which bacteria might have affected intestinal 5-hydroxytryptamine is by causing an inflammatory reaction. Zbinden and Pletscher (1958) reported that chronic irritation of the stomach and small intestine of rabbits by continuous administration of ethyl alcohol often led to an increase in the 5-hydroxytryptamine content.

It was also observed that a meat diet caused an increase in the weight of the intestine of rats. It has been reported that the small intestine in germ-free chicks weighs much less than in controls, and also that the intestinal weight could be significantly reduced by antibiotic feeding, while the body

weight of the chicks was increased (Stokstad, 1954). It was suggested that the normal animal has a chronic inflammation of the intestine. In meat-fed rats, the great increase of the anaerobic flora might, conversely, have exacerbated such chronic intestinal inflammation as might exist in the normal rat. In tryptophan-fed rats there was no increase in intestinal weight and a much smaller increase in anaerobic bacteria compared with meat-fed rats.

Eber and Lembeck (1958) reported a significant difference in the intestinal 5-hydroxytryptamine content of groups of rats fed on tryptophan-rich and tryptophan-poor diets. In the experiments described here, the addition of tryptophan to the diet had no effect on the 5-hydroxytryptamine content of rat tissues, but the controls did not receive a tryptophan-poor diet, which perhaps accounts for the lack of any difference between control and treated rats. A possible reason for the poor response of tissue 5-hydroxytryptamine levels to diets rich in tryptophan is that hydroxylation proceeds at a maximal rate even on a normal tryptophan intake (Udenfriend, Titus, Weissbach, and Peterson, 1956). Tryptophan-deficient diets are reported to cause significant reduction in intestinal 5-hydroxytryptamine in a number of species (Eber and Lembeck, 1958; Zbinden, Pletscher, and Studer, 1958) although these authors stress that the decrease in the 5-hydroxytryptamine content develops slowly.

A marked species difference was found in the relative effects in rats and mice of a tryptophan-rich diet. While no effect was produced in rats, there was a widespread rise of the 5-hydroxytryptamine content in mice, affecting the small intestine, spleen and blood platelets. Such a generalized effect on the 5-hydroxytryptamine content of mice suggests that the diet caused an increase in 5-hydroxytryptamine synthesis in these animals.

Tryptophan feeding did not affect the brain concentration of 5-hydroxytryptamine, whereas Zbinden *et al.* (1958) found that brain 5-hydroxytryptamine was reduced in rabbits, guinea-pigs, rats and mice which received a tryptophan-deficient diet.

The observation of Cordier (1926) that the number of argentaffin cells in mice was increased by meat-feeding was not confirmed in these experiments, nor was any increase found in argentaffin cells of rats as a result of meat-feeding. Zbinden and Pletscher (1958) found that in rabbits the increase in 5-hydroxytryptamine caused by ethyl alcohol was only in some instances accompanied by a moderate rise in the number of argentaffin cells. Zbinden *et al.* (1958) found that only in severe tryptophan deficiency were the histochemical reactions of the argentaffin cells diminished. It seems, therefore, that effects upon intestinal 5-hydroxytryptamine are not commonly associated with changes in the argentaffin cell count. This is confirmed by Benditt and Wong (1957), who found that a relatively high 5-hydroxytryptamine concentration, greater than 1 mg./ml., is required before argentaffin cells can be stained by silver and azo dyes.

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THE DISTRIBUTION OF 5-HYDROXYTRYPTAMINE AND ADENOSINETRIPHOSPHATE IN CYTOPLASMIC PARTICLES OF THE DOG'S SMALL INTESTINE

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The distribution of 5-hydroxytryptamine, adenosinetriphosphate, and succinic dehydrogenase in sucrose homogenates of the dog's small intestine has been studied. The adenosinetriphosphate was present in two different layers which could be separated by density gradient centrifugation. The upper layer contained also much succinic dehydrogenase, but no amine; it is probably composed of mitochondria. The lower layer contained not only adenosinetriphosphate but also the major portion of the particle-held 5-hydroxytryptamine. The mean molar ratio, amine : adenosinetriphosphate, in the lower layer was 2.6. The experiments suggest that adenosinetriphosphate in the intestine is of importance in the storage of 5-hydroxytryptamine, resembling the function of adenosinetriphosphate in the storage of the catechol amines of the adrenal medulla.

Many observations suggest that the pharmacologically active amines are stored in specific cell organelles which can be sedimented by centrifugation of tissue homogenates prepared in isotonic sucrose. Earlier work from this laboratory has shown that some of the 5-hydroxytryptamine present in homogenates of the dog duodenal mucosa can be sedimented by the use of a sucrose density gradient; by this method a considerable separation of mitochondria and 5-hydroxytryptamine-storing elements has been achieved (Baker, 1958, 1959).

In their storage location, amines seem to be associated with acidic compounds. For instance, in the mast cells histamine is found together with heparin, and in the chromaffin granules of the adrenal medulla the catechol amines occur together with adenosinetriphosphate. The work of Born (1956a ; 1956b) and of Born and Gillson (1957) suggests that adenosinetriphosphate is also associated with 5-hydroxytryptamine in the platelets.

In most mammalian species the bulk of the 5-hydroxytryptamine is found in the epithelial lining of the gastro-intestinal tract. Information on the intracellular localization of the amine in this tissue is still far from complete. This is because the cells which carry the 5-hydroxytryptamine represent only a small fraction of those present in the mucous membrane. It seems likely, therefore, that the granular material rich in 5-hydroxytryptamine as prepared by Baker (1959) is still heterogeneous, with the elements carrying the amine contaminated by other structures. In the present work an attempt has been made to achieve a further purification and to find out if the tissue contains adenosinetriphosphate, and how it is distributed among the fractions isolated.

METHODS

Isolation and Purification of Cytoplasmic Granular Fractions

The procedure adopted followed that of Baker (1958 ; 1959), except that pentobarbitone sodium was used instead of ether as anaesthetic. Dogs were bled, the small intestine was taken out and the mucous membrane was removed and homogenized in 0.3 M sucrose. The portion used always included the duodenum and extended in different experiments to a varying degree to the lower parts of the small intestine. Unbroken cells, nuclei and coarse cell debris were removed by low-speed centrifugation at 900 *g* for 20 min. The supernatant from the low-speed centrifugation was spun at high speed, 11,000 *g* for 30 min., in order to sediment the amine-carrying material. This sediment was resuspended in 0.3 M sucrose and layered over a sucrose density gradient prepared the previous day and spun at 100,000 *g* for 1 hr. in the swinging bucket rotor SW 39L of the Spinco

ultracentrifuge. The fractions obtained were separated by the help of the Schuster cutter and those found rich in 5-hydroxytryptamine and adenosinetriphosphate were diluted with 0.3 M sucrose, to obtain a final molarity of about 1.0 M. They were then again layered over a sucrose density gradient. After a second centrifugation at 100,000 *g* for 1 hr. the fractions were separated and analysed for 5-hydroxytryptamine and adenosinetriphosphate and also for succinic dehydrogenase activity.

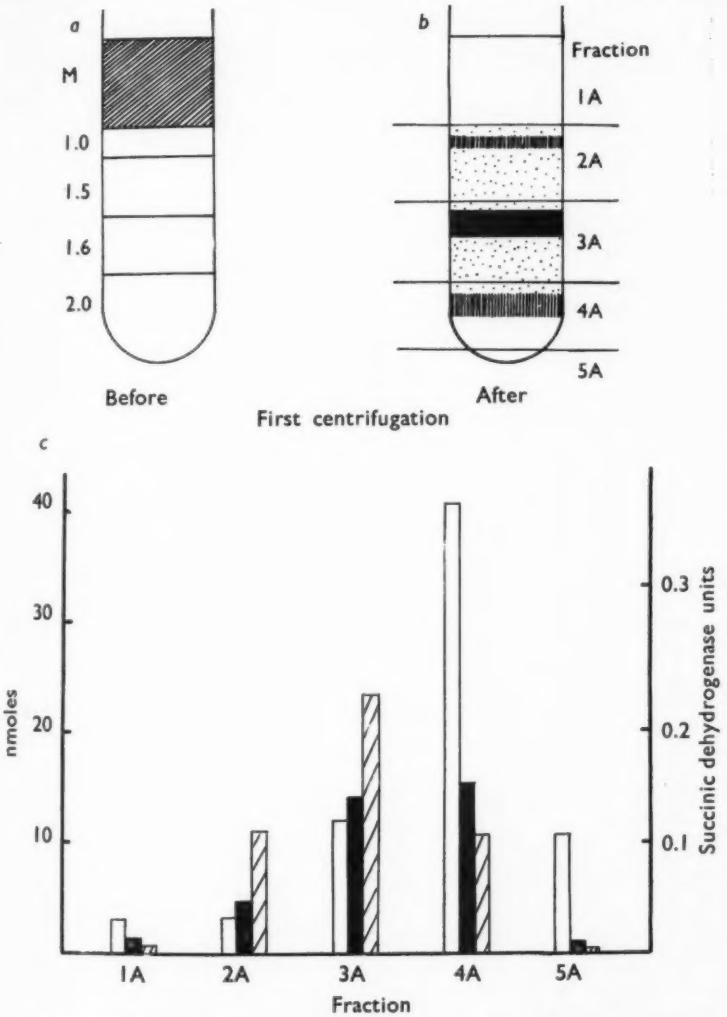


FIG. 1.—First density gradient centrifugation of cytoplasmic particles from the dog's small intestine. (1*a*) Density gradient before centrifugation, with the resuspended large cytoplasmic particles in 0.3 M sucrose at the top of the gradient (shaded area). (1*b*) Diagrammatic representation of the appearance after centrifugation for 60 min. at 100,000 *g*. The numbers at the right refer to the fractions obtained by cutting the tube. (1*c*) Distribution of 5-hydroxytryptamine (white columns), adenosinetriphosphate (black columns), and succinic dehydrogenase (shaded columns) in the particulate fractions.

The unit of succinic dehydrogenase activity is the amount of enzyme that will give an increase in optical density of 1.0 in 10 min.

Methods of Analysis

5-Hydroxytryptamine was extracted with *n*-butanol by the method of Udenfriend, Weissbach, and Clark (1955), as modified by Cargill-Thompson, Hardwick, and Wiseman (1958) and Baker (1959), and assayed on the rat gastric fundus preparation (Vane, 1957). Adenosinetriphosphate was estimated by the firefly luminescence method, as used by Holton (1959). Succinic dehydrogenase activity was determined by measuring the rate of reduction of cytochrome C, as described by Kuff and Schneider (1954).

RESULTS

The separation of the resuspended cytoplasmic particles in a sucrose density gradient is shown in Fig. 1a and 1b. The gradient was adapted from that described by Baker (1959), but the intermediate steps were chosen so as to make the distance between the different particulate fractions convenient for separation. There were three main particulate fractions, one a few mm. below the top of the gradient (2A), a second near the boundary of the 1.5 M and the 1.6 M sucrose (3A), and a third layer below the 1.6 M sucrose (4A).

The distribution of the 5-hydroxytryptamine, adenosinetriphosphate and succinic dehydrogenase in the various fractions is shown in Fig. 1c. The distribution of succinic dehydrogenase and of 5-hydroxytryptamine was essentially as found by Baker (1959); the mitochondrial enzyme had a maximum in fraction 3A, whereas the 5-hydroxytryptamine had a maximum in fraction 4A. The uppermost granular layer (2A) was poor in both enzyme and 5-hydroxytryptamine.

The distribution of adenosinetriphosphate differed from that of the two other constituents. Little was recovered in fraction 2A; the adenosinetriphosphate present was approximately equally divided between the other two particulate

fractions, 3A and 4A. In other experiments the mitochondria (3A) contained up to 3 times the amount of adenosinetriphosphate found in the dense particulate fraction (4A).

In order to determine whether adenosinetriphosphate was associated with the 5-hydroxytryptamine-containing granules, experiments were performed in which the fraction rich in 5-hydroxytryptamine (fraction 4A of Fig. 1b) was diluted with 0.3 M sucrose and again centrifuged in a sucrose density gradient. The gradient used in this centrifugation is shown in Fig. 2a, and the appearance of the gradient tube at the end of the centrifugation in Fig. 2b. The gradient differed from that used in the first gradient centrifugation in that the layer of 1.0 M sucrose at the top of the gradient was omitted. This layer was unnecessary, since the molarity of the resuspended particulate material was adjusted to approximately 1.0 M. Equal volumes of 1.5 M, 1.6 M, and 2.0 M layers of sucrose were used (Fig. 2a).

The distribution of the particulate elements after a centrifugation for 1 hr. at 100,000 *g* is shown in Fig. 2b. There were two layers, of which the larger one, fraction 4B, was present in a level equal in density to that from which the material had been obtained in the first density gradient centrifugation (fraction 4A of Fig. 1b).

Fig. 2c shows the distribution of the components in the different layers. In this figure the % distribution of the material recovered is given. It can be seen that fraction 4B was again rich in both adenosinetriphosphate and 5-hydroxytryptamine, but succinic dehydrogenase activity was still associated with this fraction. Unlike fraction 1A of the first density gradient, the top fraction, 1B, contained about one-quarter of the 5-hydroxytryptamine and also of the succinic dehydrogenase activity; this indicates that some release had accompanied the dilution with 0.3 M sucrose of the particulate material from fraction 4A, which had been recovered in strongly hypertonic sucrose. This is analogous to observations on the chromaffin granules of the adrenal medulla (Hillarp and Nilson, 1954; Eade, 1958).

A second experiment was carried out in which use was made of the experience gained from the results just described. An initial high-speed

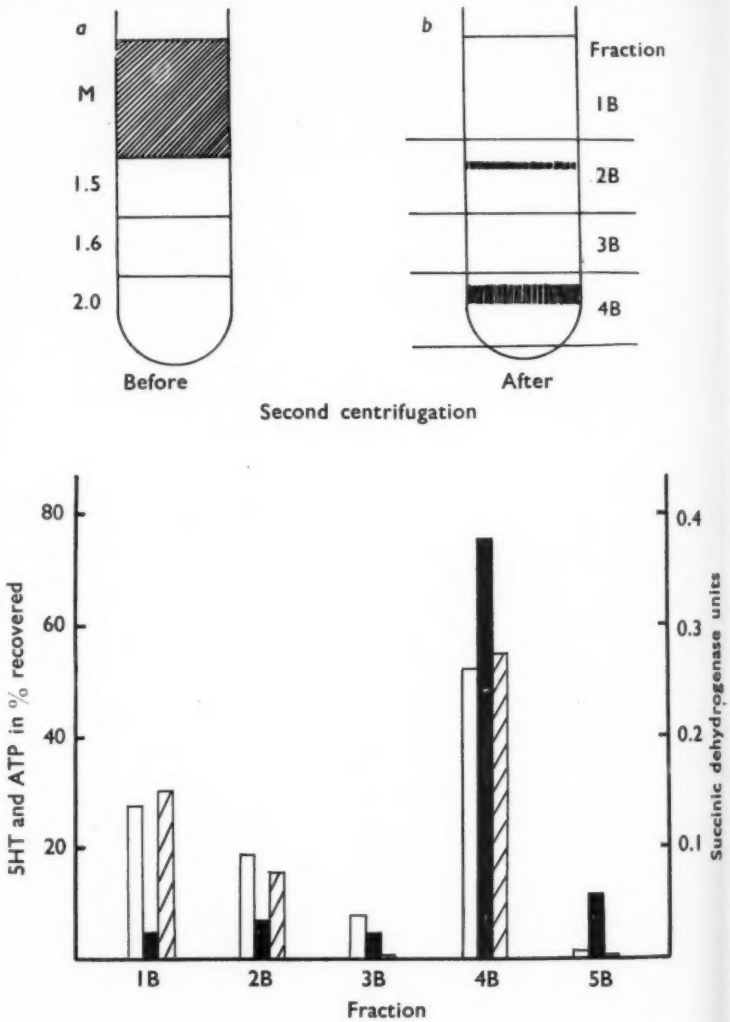
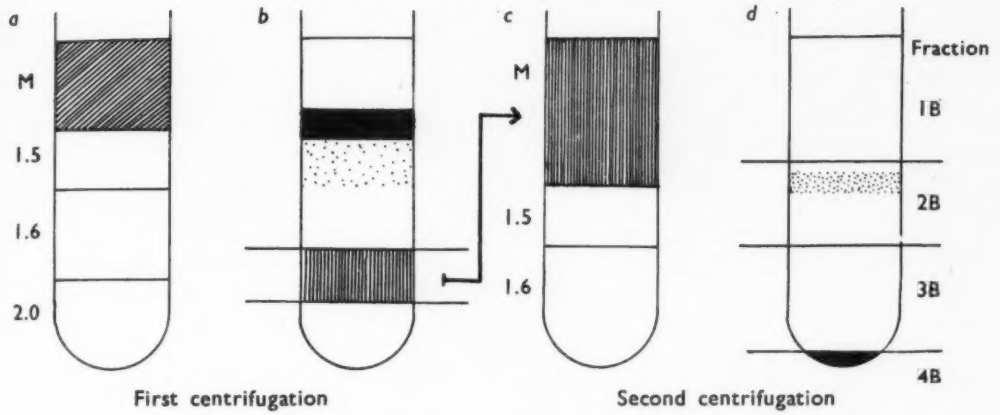


FIG. 2.—Second density gradient centrifugation of the granular fraction (4A) rich in 5-hydroxytryptamine and adenosinetriphosphate obtained in the gradient shown in Fig. 1*b*. (2*a*) Gradient before centrifugation. (2*b*) Appearance of the gradient after centrifugation for 60 min. at 100,000 *g* and position of the fractions collected. (2*c*) Determination of 5-hydroxytryptamine (white columns), adenosinetriphosphate (black columns), and succinic dehydrogenase (hatched columns) in the different fractions. Note that in this diagram 5-hydroxytryptamine and adenosinetriphosphate are expressed in terms of the total material recovered.



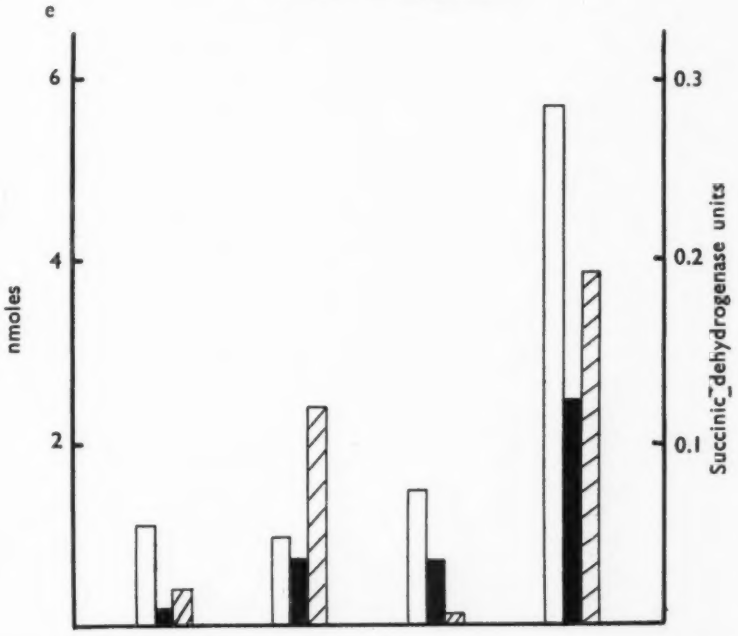


FIG. 3.—Centrifugation of dense particles obtained from the dog's small intestine. Both centrifugations were for 60 min. at 100,000 *g*. (3*a*) First density gradient before centrifugation. (3*b*) First density gradient after centrifugation. (3*c*) Second density gradient tube with the dense particulate fraction shown in 3*b* isolated during the first gradient centrifugation. (3*d*) Appearance of second gradient tube after centrifugation for 60 min. at 100,000 *g*. (3*e*) Distribution of 5-hydroxytryptamine (white columns), adenosinetriphosphate (black columns), and succinic dehydrogenase (hatched columns) in the fractions obtained after the second gradient centrifugation.

centrifugation in 0.3 M sucrose was again followed by two density gradient centrifugations of the resuspended high-speed sediment. The first sucrose gradient (Fig. 3a) was set up with 1.5 M, 1.6 M, and 2.0 M sucrose. The volumes were chosen so as to obtain a separation of the dense layer rich in 5-hydroxytryptamine from the other particulate layers of lower density. Fig. 3b shows that a satisfactory separation of this layer in the lower part of the tube was achieved.

The dense layer obtained in the first density gradient centrifugation was isolated and again diluted with 0.3 M sucrose to a molarity of 1.0 M. This suspension was layered over a second density gradient as shown in Fig. 3c. The gradient did not contain a layer of 2.0 M sucrose at the bottom of the tube; this layer was omitted in order to obtain a sedimentation of particulate material of highest density.

Fig. 3d shows that a sediment was obtained in the second density gradient centrifugation. As in the preceding experiment some opaque material was also present at the top of the gradient.

Fig. 3e shows the composition of tube at the end of the second gradient centrifugation. The sediment, which probably contributed only 1% of the total volume of the tube, contained 63% of the total 5-hydroxytryptamine and also 63% of the total adenosinetriphosphate. The sedimentation of both 5-hydroxytryptamine and adenosinetriphosphate clearly shows that both substances were associated with a particulate fraction. It may be noted that the sediment also contained some succinic dehydrogenase activity.

Molar Ratio, 5-Hydroxytryptamine : Adenosinetriphosphate

Three experiments were carried out in which 5-hydroxytryptamine and adenosinetriphosphate were determined in the fractions obtained after the first density gradient centrifugation. The mean figure for total 5-hydroxytryptamine, as recovered in all these fractions, was 8.7 ± 2.8 nmoles/g. of fresh tissue; the corresponding figure for adenosinetriphosphate was 6.0 ± 2.1 nmoles: this gives a molar ratio of 1.5. The mean 5-hydroxytryptamine content of the dense particle fraction was 4.1 ± 0.9 nmoles, the mean adenosinetriphosphate content 1.6 ± 0.4 nmoles; thus the mean molar ratio was 2.6. This increase in the molar ratio is accounted for by the fact that some adenosinetriphosphate was removed without a concomitant loss of 5-hydroxytryptamine. It seems likely that the adenosinetriphosphate removed was present in mitochondrial elements.

Discussion

The work reported in this paper confirms and extends previous observations, according to which a substantial part of the intestinal 5-hydroxytryptamine is present in a structural element that can be sedimented by high-speed centrifugation of a sucrose homogenate. Density gradient centrifugation was successful in achieving a partial separation of the particle-held 5-hydroxytryptamine from succinic dehydrogenase, but even with the higher degree of purification now achieved some enzymic activity was still found in the fraction that was rich in 5-hydroxytryptamine. Preliminary studies by electron microscopy of the fractions obtained revealed the presence of fragmented mitochondria; it is probable, therefore, that the succinic dehydrogenase is associated with these fragments.

The main interest of the present experiments lies in the fact that the fractions rich in 5-hydroxytryptamine invariably also contained similar amounts of adenosinetriphosphate. In the adrenal medulla, molar ratios of amine: adenosinetriphosphate of about 4 have been reported, and it is of interest that the figure of approximately 3 found in the present experiments is not very different from the ratio in the adrenal medulla. At the present stage of our knowledge the mechanism by which the 5-hydroxytryptamine is held at its site of storage is unknown, but the present experiments show that enough adenosinetriphosphate is present to assign to it a role similar to that which it plays in the storage of the catechol amines in the chromaffin tissue and in the adrenergic neurone.

I am grateful to Dr. H. Blaschko for the hospitality extended to me during my stay in Oxford and to Mrs. Rosemary Bonney for skilful help with the assays.

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THE ANTICHOLINESTERASE ACTIVITY OF SOME ANTIADRENALINE AGENTS

BY

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The contractions of the isolated guinea-pig vas deferens in response to stimulation of the sympathetic hypogastric nerve were potentiated by low concentrations and inhibited by high concentrations of the antiadrenaline agents tolazoline, yohimbine, ergotamine, phenoxybenzamine and piperoxan. Eserine potentiated the contractions of the vas deferens produced by hypogastric nerve stimulation. The cholinesterase activity of an extract of vas deferens was decreased by the antiadrenaline agents. The potentiation of responses to sympathetic stimulation by antiadrenaline drugs, which also possess anticholinesterase activity, can be explained on the basis of a cholinergic sympathetic mechanism.

The effects of antiadrenaline agents on the responses to stimulation of a sympathetic nerve do not always run parallel to their effects on responses induced by noradrenaline. Thus Varagić (1956b) found that tolazoline potentiated the response of the rabbit uterus to sympathetic nerve stimulation, and Huković (1959) reported that phenoxybenzamine increased the response to sympathetic nerve stimulation in the isolated atria of the rabbit.

We have investigated the effects of tolazoline and other antiadrenaline agents on the responses of the isolated vas deferens to sympathetic nerve stimulation, noradrenaline and acetylcholine, and on the cholinesterase activity of the vas deferens.

METHODS

Isolated Innervated Vas Deferens.—The isolated vas deferens with intact hypogastric nerve was prepared by the method of Huković (personal communication). Guinea-pigs weighing about 500 g. were killed by a blow on the head. The abdomen was opened in the midline and the distal colon retracted to one side. The hypogastric nerves were identified and dissected free. The vasa deferentia were cut from their attachments to the epididymis at one end and the urethra at the other and removed, each with its accompanying nerve. The vas deferens was mounted in a 50 ml. organ bath containing McEwen's (1956) solution gassed with 95% oxygen and 5% carbon dioxide and maintained at 29°. The nerve was passed through a stimulating electrode of the type described by Burn and Rand (1960a) consisting of a 1 mm. tube containing 2 adjacent platinum rings. Stimulation was by 2 msec. pulses at a frequency of 10/sec. applied at supramaximal strength (usually 3.0 mA.) for 10 sec. in every 2 min. The time cycle of stimulation was automatically controlled. Contractions were recorded by an isotonic frontal writing lever.

Isolated Vas Deferens.—For experiments in which nerve stimulation was not required the vas deferens was suspended in a 5 ml. bath.

Cholinesterase Activity.—The vasa deferentia from a guinea-pig were ground with Tyrode solution (11 ml.) in a mortar. The whole extract was divided into 2 ml. aliquots to serve as source of enzyme in each reaction mixture. The drugs examined for anticholinesterase activity were mixed with the enzyme 10 min. before the addition of substrate and brought to 32°. Acetylcholine as substrate was added to reach a final concentration of 0.5 μg./ml. in a final volume of reaction mixture of 5 ml. At various time intervals after this, the reaction mixture was agitated and 0.1 ml. samples were withdrawn and assayed for acetylcholine on a strip of guinea-pig ileum suspended in Tyrode solution in a 5 ml. bath. A dose-response curve for acetylcholine was obtained upon the ileum at the beginning and the end of each experiment.

RESULTS

Sympathetic Stimulation.—The contractions of the isolated vas deferens in response to stimulation of the sympathetic hypogastric nerve for 10 sec. at 2 min. intervals maintained a steady amplitude over long periods of time. The vas deferens was contracted by acetylcholine and noradrenaline.

When we attempted to block the response to sympathetic stimulation with the antiadrenaline

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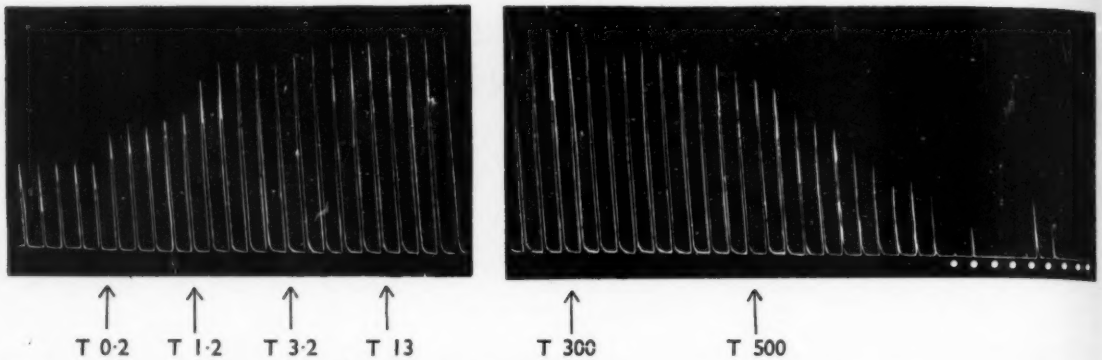

FIG. 1.—Contractions of the isolated guinea-pig vas deferens in response to hypogastric nerve stimulation at 2 min. intervals (white dots indicate stimulation not followed by contractions). At T, tolazoline was added to the bath to produce the concentrations indicated in μg./ml.

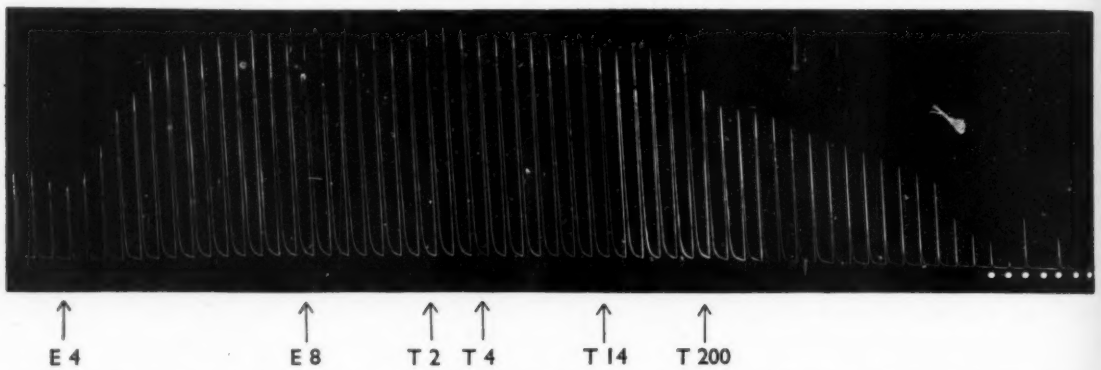

FIG. 2.—As Fig. 1. Eserine was added to the bath at E, and tolazoline at T, to produce the concentrations indicated in μg./ml.

agent tolazoline, it was observed that low doses of tolazoline produced a potentiation of the response (Fig. 1). The concentration of tolazoline required to inhibit the response to nerve stimulation was several hundred times higher than the dose which produced a clear potentiation. Thus in Fig. 1 a potentiation of the response can be seen with 0.2 μg./ml., whereas 500 μg./ml. was required to block.

In other experiments we had found that the response of the vas deferens to sympathetic nerve stimulation was potentiated by eserine. This observation led us to consider that the potentiation of the responses to sympathetic nerve stimulation that we had observed with low doses of tolazoline may be explained if it too possessed an anti-cholinesterase action. In the experiment shown in Fig. 2 the responses of the vas deferens to nerve stimulation were potentiated by eserine in a concentration of 4 μg./ml. An additional 4 μg./ml. when added to the bath had no further

TABLE I

CONCENTRATIONS OF ANTIADRENALINE AGENTS TO POTENTIATE AND TO INHIBIT RESPONSES OF VAS DEFERENS TO HYPOGASTRIC NERVE STIMULATION

	Concentration of Drug Required to Produce		Maximum Potentiation %
	Potentiation (μg./ml.)	Block (μg./ml.)	
Tolazoline	0·1–100	500	280
Phenoxybenzamine	1·0–30	130	140
Yohimbine ..	0·2–1·0	30	200
Ergotamine ..	3·0–30	70	150
Piperoxan	0·2–20	100	150

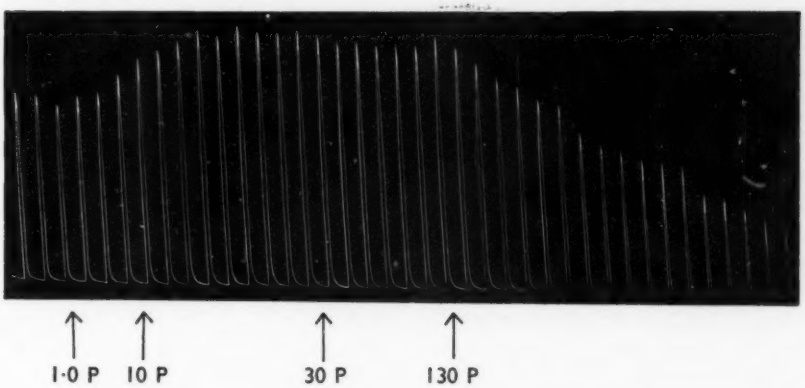

FIG. 3.—As Fig. 1. Phenoxybenzamine was added to the bath at P to produce the concentrations indicated in μg./ml.

effect, thus showing that the potentiation due to inhibition of cholinesterase was complete. Gradually increasing concentrations of tolazoline were next added to the bath without causing any further potentiation of the responses.

The effect of low doses of other antiadrenaline agents was tested on the responses of the vas deferens to hypogastric nerve stimulation. The results obtained with phenoxybenzamine are shown in Fig. 3. The responses were potentiated by a low dose of phenoxybenzamine (1.0 μg./ml.), a higher dose (30 μg./ml.) had no further effect, and finally at a still higher dose (130 μg./ml.) the responses gradually decreased until there was complete block. Similar results were obtained for yohimbine, piperoxan and ergotamine. The findings are summarized in Table I.

Noradrenaline and Acetylcholine.—The contractions of the isolated vas deferens in response to acetylcholine added to the bath were potentiated by tolazoline, but the responses to noradrenaline showed no sign of potentiation. Thus in Fig. 4, 30 μg./ml. of tolazoline inhibited the response to noradrenaline but potentiated the response to

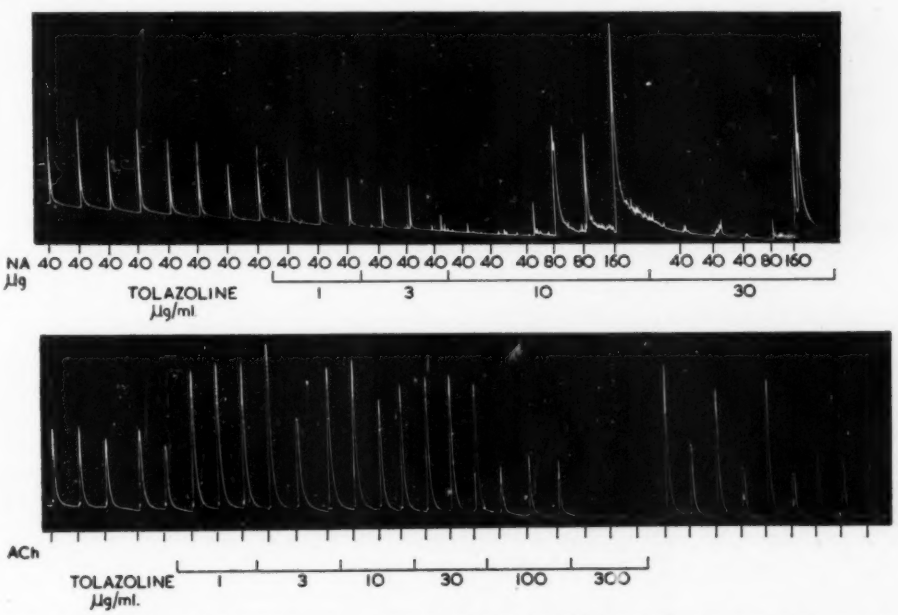

FIG. 4.—Effect of tolazoline on responses of the isolated guinea-pig vas deferens to noradrenaline and acetylcholine. In the upper tracing, the responses to 40 μg. of noradrenaline were abolished by 10 μg./ml. of tolazoline, although higher doses of noradrenaline (80 μg. and 160 μg.) were still effective. In the lower tracing the responses to acetylcholine (5 μg.) were potentiated by 1 to 30 μg./ml. of tolazoline, and inhibited by 300 μg./ml.

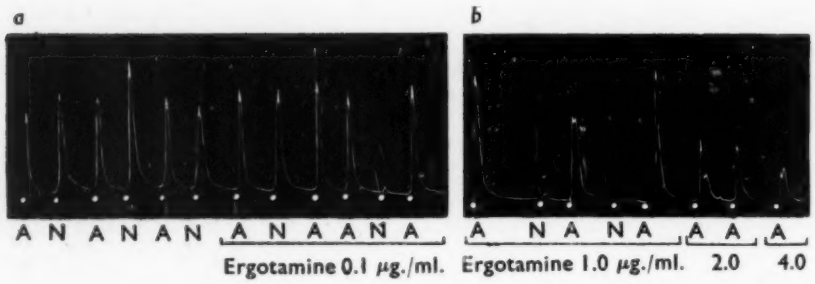

FIG. 5.—Effect of ergotamine on responses of the isolated guinea-pig vas deferens to acetylcholine (A, 0.2 μg./ml.) and noradrenaline (N, 2 μg./ml.).

acetylcholine. The block of noradrenaline by tolazoline was readily overcome by larger doses of noradrenaline.

Ergotamine in concentrations of 0.1 to 1.0 μg./ml. potentiated the responses to acetylcholine as shown in Fig. 5. In higher concentrations (2.0 to 4.0 μg./ml.), ergotamine exhibited an atropine-like action in diminishing the responses to acetylcholine. Similar results were obtained with phenoxybenzamine, piperoxan and yohimbine; low concentrations potentiated the contractions produced by acetylcholine, while higher concentrations reduced them.

We observed no potentiation of the contractions of the vas deferens in response to noradrenaline with ergotamine (Fig. 5), phenoxybenzamine, piperoxan or yohimbine.

Atropine and Eserine.—It is apparent that the antiadrenaline agents which we have investigated possess an atropine-like action in that they depress the response of the vas deferens to acetylcholine.

Our findings with tolazoline had led us to suppose that the potentiation of the responses of the vas deferens to hypogastric nerve stimulation could be attributed to its anticholinesterase activity. Since the antiadrenaline agents potentiated the responses to hypogastric nerve stimulation, but in addition possessed an atropine-like action, we investigated the effect of atropine on the potentiation of the responses to hypogastric nerve stimulation produced by eserine. In Fig. 6 the responses to hypogastric nerve stimulation were reduced in the presence of 0.1 μg./ml. of atropine, which was a sufficient dose to block completely the response to acetylcholine. Eserine (4 μg./ml.) when added to the bath still potentiated the responses to sympathetic nerve stimulation.

Fig. 7 illustrates an experiment in which dibenzyline produced a potentiation of the responses of the vas deferens to nerve stimulation although the dose of phenoxybenzamine used was sufficient to abolish the responses to both acetylcholine and noradrenaline.

Anticholinesterase Activity.—There were grounds for considering that the action of low doses of the antiadrenaline agents in potentiating the responses of the vas deferens to hypogastric nerve stimulation was due to their anticholinesterase activity. We selected a biological assay method for determining residual acetylcholine in the enzyme reaction mixture since it was the only way in which we could test enzyme activity on low concentrations of substrate. Thus, the acetylcholine content of the reaction mixture was 2.3×10^{-6} M; for the manometric determination of anticholinesterase activity concentrations of 1×10^{-2} M have generally been employed.

Tolazoline was the most effective of the drugs tested in inhibiting the cholinesterase of guinea-pig vas deferens. Fig. 8 shows that inhibition was detectable with 1 μg./ml., appreciable with 10 μg./ml., and complete with 500 μg./ml. Ergotamine (100 μg./ml.) produced a 35% inhibition

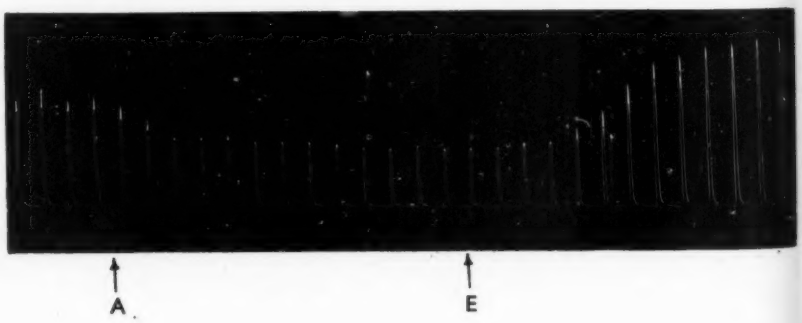

FIG. 6.—As Fig. 1. At A, atropine was added to the bath to produce a concentration of 0.1 μg./ml. At E, eserine was added to produce a concentration of 4 μg./ml.

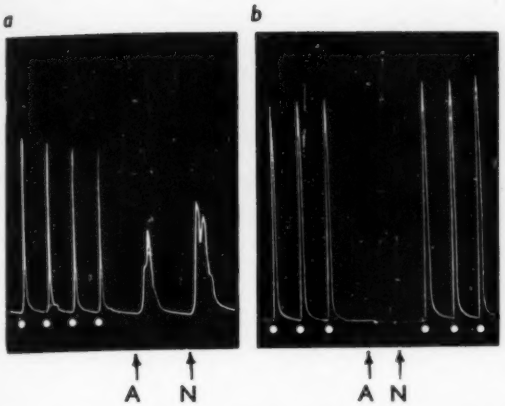

FIG. 7.—Isolated guinea-pig vas deferens. At white dots the hypogastric nerve was stimulated. At A, 10 μg./ml. of acetylcholine, and at N, 10 μg./ml. of noradrenaline was added. Between tracings (*a*) and (*b*) phenoxybenzamine was added to produce a bath concentration of 3 μg./ml.

of cholinesterase activity measured over the first 10 min. of the reaction.

Piperoxan and yohimbine were approximately equiactive; both produced a 30% inhibition of enzyme activity in concentration of 100 μg./ml. Phenoxybenzamine was the least active; 50 μg./ml. producing a 10% inhibition, and 500 μg./ml. a 25% inhibition during the first 10 min. of enzyme action.

DISCUSSION

Jang (1941) attributed the increase in response to sympathetic stimulation produced by ergotamine, piperoxan, and yohimbine to their action in enhancing the response to adrenaline. He found that small doses of these three drugs increased the vasoconstriction in the rabbit ear produced by adrenaline. Other reports occur in the literature of potentiating action towards adrenaline and noradrenaline of antiadrenaline agents when they are used in low doses. Thus Holzbauer and Vogt (1955) reported that dibenamine, phenoxybenzamine, and dihydroergotamine made the rat uterus more sensitive to the inhibitory action of adrenaline, although tolazoline, yohimbine, piperoxan, and phentolamine had no such effect. In order for Jang's suggestion to hold good it would be necessary to show that the antiadrenaline drugs which potentiated the response of the vas deferens to sympathetic stimulation were effective in potentiating the responses to noradrenaline. We have not observed any increase in response to noradrenaline in the presence of antiadrenaline agents.

The potentiation of the responses to sympathetic stimulation produced by low concentrations of antiadrenaline agents can be understood in the light of their anticholinesterase action. There are two ways in which an inhibition of the

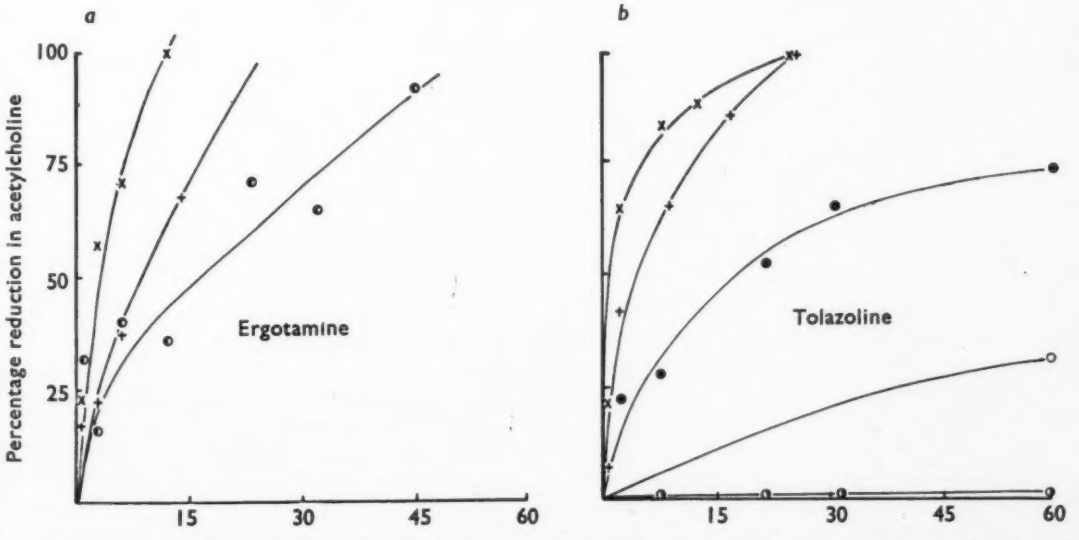

FIG. 8.—The rate of the destruction of acetylcholine by an extract of guinea-pig vas deferens in the presence of various concentrations of ergotamine (*a*) and tolazoline (*b*). X=Control. +=1 μg./ml. ●=10 μg./ml. O=50 μg./ml. ◐=100 μg./ml. ◑=500 μg./ml. Ordinate: % reduction in acetylcholine concentration Abscissa: time in min.

destruction of acetylcholine from cholinergic sympathetic fibres could increase the responses of the vas deferens to hypogastric nerve stimulation: by increasing the direct action of acetylcholine on effector cells, or its intermediary action in liberating noradrenaline. The mechanism by which acetylcholine and cholinergic sympathetic fibres might liberate noradrenaline has been discussed by Burn and Rand (1959, 1960b). The direct action of acetylcholine on the vas deferens was blocked by atropine, but inhibition of cholinesterase by eserine still led to potentiation of the responses to sympathetic stimulation (Fig. 6) which favours the idea that it is the action of acetylcholine as an intermediary in noradrenaline release that is being potentiated. Thus ergotamine, phenoxybenzamine, piperoxan, and yohimbine, which had atropine-like activity, nevertheless potentiated the responses to sympathetic nerve stimulation.

The potentiation of the responses to sympathetic stimulation by anticholinesterase drugs has been observed in the nictitating membrane (Bacq and Fredericq, 1935a), in the rabbit ear (Burn and Rand, 1960b), in some experiments in the rabbit uterus (Varagić, 1956b) and by us in the guinea-pig vas deferens.

Potentiation of responses to sympathetic stimulation by low concentrations of anti-adrenaline drugs has been observed in isolated rabbit atria with dibenzyline (Huković, 1959), in the isolated rabbit uterus with tolazoline (Varagić, 1956b), in the cat nictitating membrane with piperoxan (Bacq and Fredericq, 1935b) and with yohimbine and ergotoxine (Jang, 1941), and in the rabbit ear with piperoxan (Jang, 1941).

Anticholinesterase activity has been reported previously for ergotamine with horse blood as the source of enzyme (Matthes, 1930), with horse serum (Gautrelet and Scheiner, 1939), with defibrinated cat blood (Brügger, 1938), and with human serum (Thompson, Tickner, and Webster, 1955). Schär-Wüthrich (1943) found that tolazoline (and two other imidazolines) inhibited cholinesterase from human serum and brain. Some actions of tolazoline can be interpreted as due to its inhibition of cholinesterase. For example, tolazoline decreased the rate of isolated rabbit atria and potentiated acetylcholine-induced contraction in the frog rectus and guinea-pig ileum (Gowdey, 1948). The gastro-intestinal stimulating action and the effect on heart rate could be prevented by atropine (Ahlquist, Huggins, and Woodbury, 1947).

Varagić (1956a) found that tolazoline altered the response of the isolated rabbit colon to sympathetic stimulation from a simple inhibition to a biphasic response consisting of an initial contraction followed by relaxation. This observation could not be explained in terms of an antagonism or reversal of the inhibition by tolazoline since Ahlquist *et al.* (1947) showed that tolazoline did not influence the inhibitory action of adrenaline, and Gowdey (1948) found tolazoline reduced but did not reverse the inhibitory action of adrenaline on the rabbit duodenum. Gillespie and Mackenna (1959) found that rabbit colon taken from a reserpine-treated rabbit contracted in response to sympathetic stimulation, and the contraction was abolished by atropine. This can be construed as evidence for cholinergic nerves running in the sympathetic supply to the colon. The action of the acetylcholine liberated from such nerves might be enhanced by an anticholinesterase so that the normal colon would be exposed to the combined effect of acetylcholine and noradrenaline to result in the biphasic response observed by Varagić (1956a). In fact, Varagić observed that eserine only produced a biphasic response in 2 of 11 preparations, but that eserine could enhance the effect of tolazoline. The initial contraction of the colon in response to sympathetic stimulation in the presence of tolazoline was abolished by atropine.

Brown and Gillespie (1957) found that the amount of noradrenaline appearing in the splenic venous blood after stimulating the splenic nerves was increased in the presence of phenoxybenzamine. Huković (1959) suggested that the appearance of a greater amount of transmitter (noradrenaline) might explain both his finding that phenoxybenzamine increased the effect of sympathetic stimulation on the rabbit atria, and Varagić's (1956b) finding that the response of the rabbit uterus to sympathetic stimulation was increased by tolazoline. We believe that an increased amount of transmitter could be released in the presence of an anticholinesterase (such as phenoxybenzamine or tolazoline) if there were a cholinergic process involved in the release of noradrenaline. Such a mechanism has been proposed by Burn and Rand (1959, 1960b) and additional evidence for it has been obtained recently when it was found that hemicholinium produced transmission failure in isolated vas deferens stimulated via its hypogastric sympathetic nerve (Rand and Chang, 1960).

Goodman and Gilman (1956) have pointed out that, "Practically every important class of drug and innumerable compounds of lesser interest have been tested for their effects on cholinesterase activity. The drugs reported to inhibit various cholinesterases outnumber those that are

apparently ineffective. Numerous exorbitant claims have been made that certain agents act principally through cholinesterase inhibition." Nevertheless the use of antiadrenaline agents as tools for investigating mechanisms must be evaluated in the light of our findings that they possess anticholinesterase activity. Thus Burnstock (1958) found that piperoxan increased the tone of longitudinal muscle in the trout stomach. He attributed this to the antagonism of an inhibitory action of adrenaline on the gut and the resultant was an acetylcholine-induced spasm. Our results show that another explanation may be the potentiation of acetylcholine by virtue of the anticholinesterase activity of piperoxan.

Finally, we wish to point out that we have no evidence to suggest that the antagonistic action of antiadrenaline agents is associated with their anticholinesterase activity.

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THE EFFECTS OF CORTICOSTEROIDS AND RELATED COMPOUNDS ON THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT TISSUES

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Daily intramuscular injections of cortisone, prednisolone, triamcinolone, dexamethasone, fludrocortisone and 2-methylfludrocortisone markedly reduced the histamine and 5-hydroxytryptamine contents of the skin and small intestine of rats; there was an increase, however, in the amine contents of the stomach. The sex hormones were weaker in these effects while deoxycortone and corticotrophin were inactive. When the skin was depleted of half of its 5-hydroxytryptamine by prolonged treatment with polymyxin B, the rate of recovery of this amine was retarded by the glucocorticoids. The glucocorticoid activity of a steroid and its ability to produce changes in the histamine and 5-hydroxytryptamine contents of tissues are related.

A functional relationship between histamine and the adrenal cortex was postulated by Dale in 1920 when he found that after adrenalectomy cats became about five times more sensitive to intravenous injections of histamine. A similar result has since been obtained by other workers using different species. The histamine contents of rat tissues are lowered after treatment with adrenal cortical steroids and raised after adrenalectomy (Schayer, Smiley, and Davis, 1954; Halpern and Briot, 1956). Recently, the 5-hydroxytryptamine contents of tissues have been shown to be similarly altered, and thus the secretion of the adrenal cortex may exert a functional control over the tissue reserves of histamine and 5-hydroxytryptamine (Hicks and West, 1958a and 1958b). The present work was undertaken to test this hypothesis further by using more potent corticosteroids than those used by previous authors.

METHODS

Groups of 4 female albino rats (120 to 180 g.) were used in all experiments. They were fed on No. 41B cubes (Associated London Flour Millers) and allowed drinking water *ad libitum*. The temperature of the room in which the animals were housed was $70 \pm 1°$ F.

Drug Treatment.—Aqueous suspensions of the corticosteroids (10 mg./kg.) were injected daily by the intramuscular route. Cortisone acetate, prednisolone, fludrocortisone, triamcinolone, and deoxycortone acetate were given for 4 and 9 days, whilst dexamethasone and 2-methylfludrocortisone (9α-fluoro-11β,17α, 21-trihydroxy-2α-methylpregn-4-ene-3,20-dione) were given only for 4 days. The formulae of these steroids are shown in Fig. 1. Intramuscular injections of oily solutions of oestradiol monobenzoate, stilboestrol and testosterone propionate (10 mg./kg.) were given on alternate days for 21 days. Long-acting corticotrophin (A.C.T.H., Organon, batch No. 6991) was injected intramuscularly for 9 days in daily doses of 1, 2, and 5 i.u. per rat. Groups of control rats received the corresponding volumes of the suspending fluid, arachis oil, or saline. The animals were killed 24 hr. after the last injection.

Depletion of Tissue Amines.—Repeated intraperitoneal injections of polymyxin B were used to produce maximal depletion of histamine in tissues rich in mast cells (for example, the skin). This treatment also lowers the 5-hydroxytryptamine content of the skin by about 50% (Telford and West, 1960). One group of polymyxin-treated rats was killed 24 hr. after the last injection, while other polymyxin-treated groups received daily doses of either saline or cortisone, prednisolone or deoxycortone (10 mg./kg.) for 9 days before being killed.

Extraction of Tissues and Assay Procedures for Histamine and 5-Hydroxytryptamine

The tissues studied in detail were abdominal skin, ears, dorsal skin of the hind feet, jejunum and pyloric stomach. On occasion, the fundic stomach duodenum, ileum, colon and lung were also used, and in some experiments with prednisolone the whole animal was extracted. Samples of the tissues from

Corticosteroid	Abbrev.	Formula					
DEOXYCORTONE	D	$=H_2$	$=H_2$	$-H$	$=H_2$	$-H$	$=H_2$
CORTISONE	C	$=H_2$	$=H_2$	$-H$	$=O$	$-OH$	$=H_2$
PREDNISOLONE	P	$-H$ double bond $-H$		$-H$	$-H$ $-OH$	$-OH$	$=H_2$
TRIAMCINOLONE	T	$-H$ double bond $-H$		$-F$	$-H$ $-OH$	$-OH$	$-H$ $-OH$
DEXAMETHASONE	Dx	$-H$ double bond $-H$		$-F$	$-H$ $-OH$	$-OH$	$-H$ $-CH_3$
FLUDROCORTISONE	Fc	$=H_2$	$=H_2$	$-F$	$-H$ $-OH$	$-OH$	$=H_2$
2-METHYLFLUDROCORTISONE	MFc	$-CH_3$ $-H$	$=H_2$	$-F$	$-H$ $-OH$	$-OH$	$=H_2$

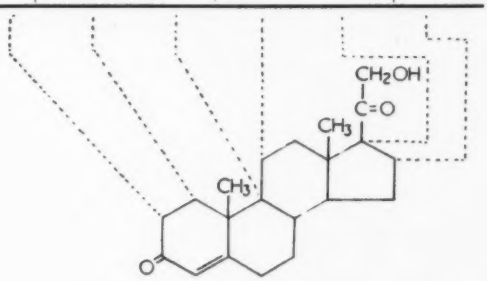

FIG. 1.—Formulae of the steroids used in the present study.

the 4 rats in each group were pooled, and either extracted with trichloracetic acid and assayed on the isolated guinea-pig ileum for histamine or extracted with acetone and assayed on the isolated rat uterus for 5-hydroxytryptamine. The methods have been described in detail elsewhere (Parratt and West, 1957). Each value of histamine and 5-hydroxytryptamine in this paper refers to the base and represents the mean of the results obtained from at least 3 separate experiments. There was considerable variation in the amounts of extractable amine from many tissues. Statistical analysis showed that values differing from the means by more than 25% were significant ($P=0.95$). The values quoted for rat skin in Table III and Figs. 2, 3, 4, 11, and 12 are the means of the 3 values for abdominal skin, ears and dorsal skin of the feet.

The steroids themselves may be extracted by the procedures used. However, concentrations of 10^{-5} of each steroid did not modify the responses of the standard amines on the isolated biological preparations.

Histological Examination. — Fresh tissue spreads from subcutaneous connective tissue, mesentery and

TABLE I

THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT (μG./G.) OF TISSUES OF CONTROL RATS

Tissue	Histamine	5-Hydroxytryptamine
Abdominal skin ..	30·1	1·9
Skin of the feet ..	66·2	1·5
Ears	37·9	1·4
Fundic stomach ..	12·6	1·8
Pyloric stomach ..	31·1	2·1
Duodenum ..	25·2	5·6
Jejunum	25·1	3·2
Ileum	23·8	5·5
Colon	17·6	3·8
Lung	8·0	3·9
Whole rat (μg.) ..	1,194·6	24·3

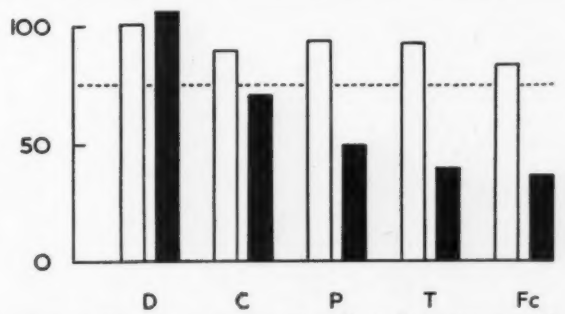

FIG. 2.—Histograms to show the effects of corticosteroids (10 mg./kg. for 4 days) on the histamine (□) and 5-hydroxytryptamine (■) contents of rat skin. All values are expressed as percentages of the control values. Values under 75% denote a loss of amine. Abbreviations of the names of the corticosteroids are as shown in Fig. 1. Note that the more active glucocorticoids depleted the 5-hydroxytryptamine but not the histamine.

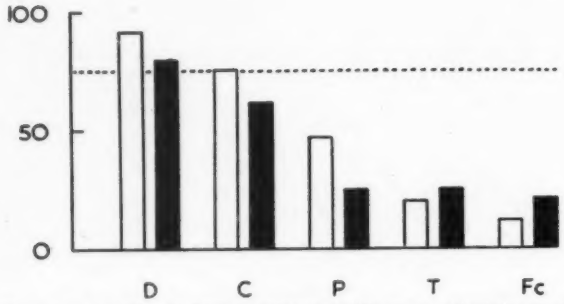

FIG. 3.—As Fig. 2, but corticosteroids were administered for 9 days. Note that the glucocorticoids depleted the skin of both 5-hydroxytryptamine and histamine.

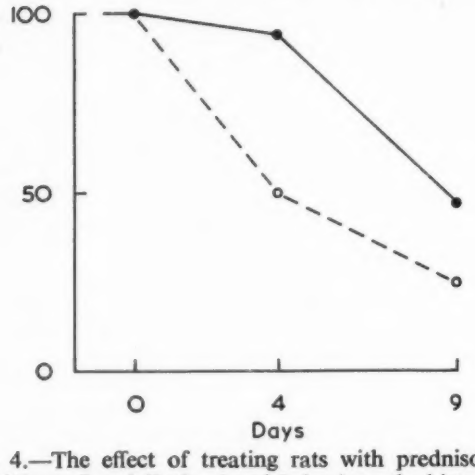

FIG. 4.—The effect of treating rats with prednisolone (10 mg./kg. daily for 4 and 9 days) on the histamine (●——●) and 5-hydroxytryptamine (O - - - O) contents of rat skin. All values are expressed as percentages of the control values. Note that 5-hydroxytryptamine was depleted before histamine.

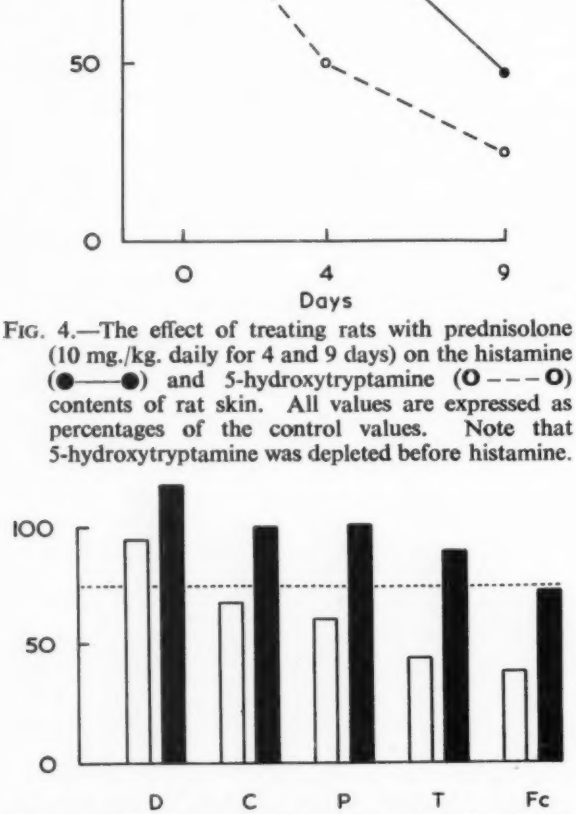

FIG. 5.—Histograms to show the effects of corticosteroids (10 mg./kg. for 4 days) on the histamine (□) and 5-hydroxytryptamine (■) contents of rat jejunum. All values are expressed as percentages of the control values. Values under 75% denote a loss of amine. Abbreviations as in Fig. 1. Note that the more active glucocorticoids depleted the histamine but not the 5-hydroxytryptamine.

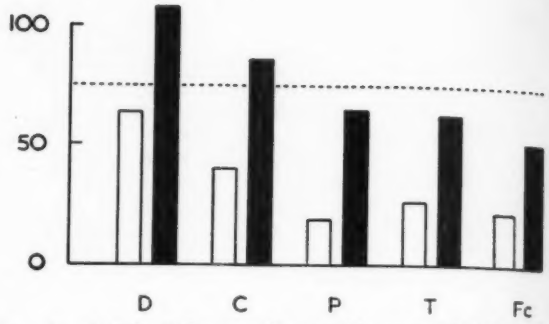

FIG. 6.—As Fig. 5, but corticosteroids were administered for 9 days. Note that the jejunum was depleted of both amines.

pleura, were fixed in dehydrated ethanol. They were then stained with toluidine blue (0.1% w/v aqueous), washed with 50% (v/v) ethanol, and taken through xylene before mounting.

RESULTS

The Histamine and 5-Hydroxytryptamine Content of Rat Tissues

The histamine and 5-hydroxytryptamine content of tissues which are rich sources of these amines in the rat is shown in Table I. The skin generally contains more histamine but less 5-hydroxytryptamine than the gut. It should be noted that the histamine content of the whole animal is about 50 times that of 5-hydroxytryptamine.

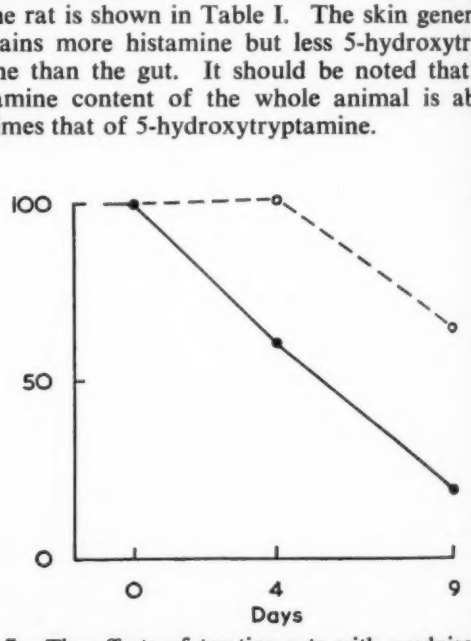

FIG. 7.—The effects of treating rats with prednisolone (10 mg./kg. daily for 4 or 9 days) on the histamine (●——●) and 5-hydroxytryptamine (O - - - O) contents of rat jejunum. All values are expressed as percentages of the control values. Note that histamine was depleted before 5-hydroxytryptamine.

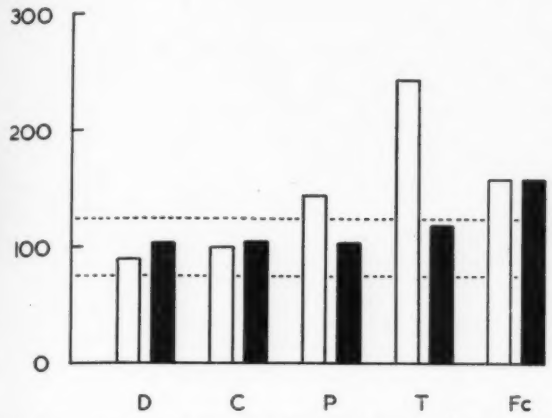

FIG. 8.—Histograms to show the effects of corticosteroids (10 mg./kg. for 4 days) on the histamine (□) and 5-hydroxytryptamine (■) contents of the pyloric stomach of rats. All values are expressed as percentages of the control values. Values over 125% denote an increase in amine content. Abbreviations as in Fig. 1. Note the increase in both amines, particularly the histamine.

The Effects of Some Corticosteroids on the Histamine and 5-Hydroxytryptamine Contents of Rat Tissues

Skin.—Treatment with the corticosteroids for 4 days produced a loss of 5-hydroxytryptamine, but not of histamine, from the skin. The most active steroids were those with the most potent glucocorticoid activity, deoxycortone (a mineralocorticoid) being ineffective (Fig. 2). When treatment was extended to 9 days, not only was there a further reduction in 5-hydroxytryptamine but the skin was also depleted of its histamine. For example, only 12% of the histamine and 21% of the 5-hydroxytryptamine remained in the skin after treatment with fludrocortisone (Fig. 3). With prednisolone the loss of skin 5-hydroxytryptamine preceded that of histamine (Fig. 4).

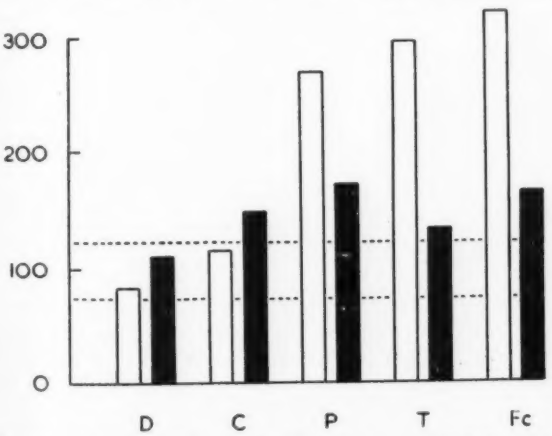

FIG. 9.—As Fig. 8, but corticosteroids were administered for 9 days.

Jejunum.—In this tissue, treatment with the corticosteroids for 4 days produced a loss of histamine but not of 5-hydroxytryptamine. As in the skin, the most active steroids were those possessing most potent glucocorticoid activity (Fig. 5). Deoxycortone was inactive. Extending the treatment to 9 days resulted in a further

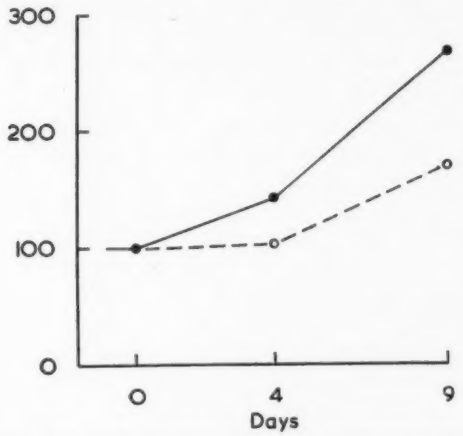

FIG. 10.—The effects of treating rats with prednisolone (10 mg./kg. daily for 4 or 9 days) on the histamine (●——●) and 5-hydroxytryptamine (O – – – O) contents of rat pyloric stomach. All values are expressed as percentages of the control values. Note the increase in both amines, particularly the histamine.

TABLE II

THE EFFECTS OF 9 DAILY DOSES OF PREDNISOLONE (10 MG./KG.) ON THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT TISSUES

All values are expressed as percentages of the control values.

Tissue	Histamine	5-Hydroxytryptamine
Abdominal skin ..	55	25
Skin of the feet ..	41	28
Ears	46	23
Fundic stomach ..	287	129
Pyloric stomach ..	270	173
Duodenum ..	19	57
Jejunum	19	65
Ileum	16	65
Colon	45	64
Lung	104	66
Whole rat	59	43

TABLE III

THE EFFECTS OF 4 DAILY DOSES OF DEXAMETHASONE, FLUDROCORTISONE AND 2-METHYLFLUDROCORTISONE (10 MG./KG.) ON THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT TISSUES

All values are expressed as percentages of the control values.

Tissue	Dexamethasone		Fludrocortisone		2-Methylfludrocortisone	
	Histamine	5-Hydroxytryptamine	Histamine	5-Hydroxytryptamine	Histamine	5-Hydroxytryptamine
Skin	85	44	84	37	111	61
Jejunum ..	37	59	38	73	38	99
Pyloric stomach	131	102	158	158	197	90

reduction in the histamine levels and a considerable loss of 5-hydroxytryptamine. For example, only 22% of the histamine and 52% of the 5-hydroxytryptamine remained in the jejunum after treatment with fludrocortisone (Fig. 6). With prednisolone, the loss of histamine in the jejunum preceded that of 5-hydroxytryptamine (Fig. 7). This alteration in the amine content was the reverse of that occurring in the skin.

Pyloric Stomach.—In striking contrast to the effects in the skin and jejunum, the histamine content of the pyloric stomach was markedly raised by treatment with the more potent corticosteroids (Figs. 8 and 9). For example, the histamine content of the pyloric stomach after 9 injections of fludrocortisone was more than 3 times the control value and the 5-hydroxytryptamine content was also raised but to a lesser degree. Deoxycortone was ineffective. The results with prednisolone are plotted graphically in Fig. 10.

Other Tissues.—The effect of prednisolone on the histamine and 5-hydroxytryptamine contents of other rat tissues is shown in Table II. The increase in the histamine content of the fundic stomach was similar to that found in the pyloric stomach, and the decrease in the histamine content of the duodenum and ileum was comparable to that found in the jejunum. The histamine content of the lung, however, was unaffected. The 5-hydroxytryptamine contents of the intestinal tissues and lung were reduced to about 60%.

The Whole Animal. — After 9 doses of prednisolone, both the histamine and the 5-hydroxytryptamine contents of the whole animal were reduced by about 50% (Table II).

The Effects of Dexamethasone and 2-Methylfludrocortisone on the Histamine and 5-Hydroxytryptamine Contents of Rat Tissues

Dexamethasone and 2-methylfludrocortisone produced changes in the tissue amines which closely followed those found after other glucocorticoids (Table III). Thus, the 5-hydroxytryptamine in the skin and the histamine in the jejunum were reduced, and the histamine in the pyloric stomach was slightly increased.

The Effects of Corticosteroids on Tissue Mast Cells

Corticosteroids produced changes in the tissue mast cells of the subcutaneous connective tissue which closely followed the release of histamine from the skin. Marked degranulation or disruption of many of the cells occurred after treatment with the more active compounds. In the mesentery, the most prominent feature was extensive swelling of the mast cells in the "windows," the size of more than one-third being about twice that of mast cells from control animals. The pleural mast

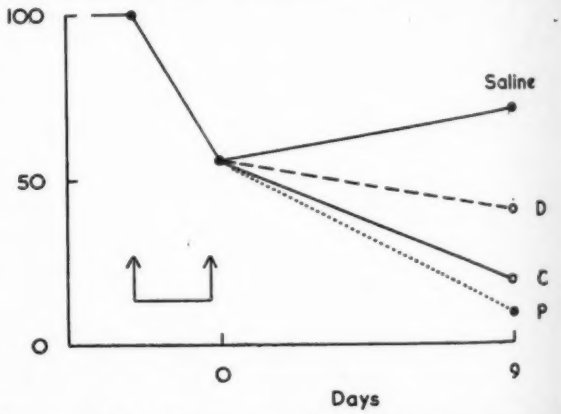

FIG. 11.—The effects of corticosteroids (10 mg./kg. for 9 days) on the recovery rate of skin 5-hydroxytryptamine, after depletion by repeated doses of polymyxin B (between the two arrows). All values are expressed as percentages of the control values. Note that the corticosteroids not only retarded the recovery of skin 5-hydroxytryptamine but lowered the value still further.

TABLE IV

THE EFFECTS OF SEX HORMONES (10 MG./KG. ON ALTERNATE DAYS FOR 21 DAYS) ON THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT TISSUES

All values are expressed as percentages of the control values.

Tissue	Oestradiol		Stilboestrol		Testosterone	
	Hist-amine	5-Hydroxy-tryptamine	Hist-amine	5-Hydroxy-tryptamine	Hist-amine	5-Hydroxy-tryptamine
Abdominal skin	91	30	94	40	93	56
Skin of the feet	93	38	104	48	85	62
Ears	65	29	113	21	91	46
Jejunum ..	110	57	87	72	78	54
Pyloric stomach	129	97	172	99	300	189

cells, however, showed little or no change after prednisolone treatment, and there was no histamine release from the lungs.

The Effect of Corticosteroids on Adrenal Weight and Body Weight

Treatment with the more active glucocorticoids for 9 days resulted in a steady loss of body weight of the animals. The loss with prednisolone, for example, was 18%, with fludrocortisone it was 21%, and with triamcinolone it was 26%. There was no change in body weight after cortisone treatment, whilst rats receiving deoxycortone gained weight at the same rate as did the control animals. The total adrenal weights, expressed as percentages of body weight, also decreased under treatment with the more active glucocorticoids.

The Effects of Corticotrophin on the Histamine and 5-Hydroxtryptamine Contents of Rat Tissues

Corticotrophin in the doses used increased the weight of the adrenal glands by over 300% but did not change either the histamine content or the 5-hydroxytryptamine content of any of the rat tissues studied.

The Effects of Sex Hormones on the Histamine and 5-Hydroxytryptamine Contents of Rat Tissues

Treatment with a natural female sex hormone (oestradiol), a synthetic female sex hormone (stilboestrol), and a male sex hormone (testosterone) lowered the skin 5-hydroxytryptamine without markedly altering the skin histamine. The changes in the amine contents of the jejunum were small, but there were increases in the histamine content of the pyloric stomach (Table IV). These changes are similar to those found after short-term treatment with the glucocorticoids.

The Effect of Corticosteroids on the Histamine and 5-Hydroxytryptamine Contents of Tissues of Rats Pre-treated with Polymyxin B

The rate of recovery of skin histamine after depletion by repeated doses of polymxin B was

TABLE V

COMPARISON OF THE EFFECTIVENESS OF CORTICOSTEROIDS IN ALLERGY AND INFLAMMATION WITH THEIR GLUCOCORTICOID AND MINERALOCORTICOID ACTIVITIES, AND WITH THEIR EFFECTS ON HISTAMINE AND 5-HYDROXYTRYPTAMINE METABOLISM

All values are expressed on a relative scale from 0 to +++.

Corticosteroid	Allergy and Inflammation	Glucocorticoid Activity	Mineralocorticoid Activity	Histamine and 5-Hydroxytryptamine Metabolism
Cortisone	+	+	0	+
Prednisolone	++	++	0	++
Fludrocortisone	++	+++	+	+++
Triamcinolone	++	+++	0	+++
Dexamethasone	++	+++	0	+++
2-Methylfludrocortisone ..	++	+++	+++	+++
Deoxycortone	0	0	+	0

not significantly altered by treatment with prednisolone, deoxycortone or cortisone. The rate of recovery of skin 5-hydroxytryptamine, however, was retarded not only by treatment with prednisolone or with cortisone but also by treatment with deoxycortone. Polymyxin reduced the skin 5-hydroxytryptamine by about 50%, and repeated doses of the corticosteroids lowered the values even further (Fig. 11).

Discussion

The pronounced changes occurring in the histamine and 5-hydroxytryptamine contents of rat tissues after injections of synthetic corticosteroids support the hypothesis of Hicks and West (1958a) that the tissue reserves of these amines may in part be regulated by the secretion of the adrenal cortex. The adrenal steroids most active in producing changes in the amine contents of tissues are those possessing greatest glucocorticoid activity, and no correlation has been found between the mineralocorticoid activity of corticosteroids and their effects on tissue amine levels (see Table V). The most potent glucocorticoids used in the present experiments, for example, are triamcinolone, dexamethasone, fludrocortisone and 2-methylfludrocortisone, and these are the most potent depletors of histamine and 5-hydroxytryptamine from the skin and small intestine. Prednisolone is weaker in these respects, yet it is more active than cortisone; deoxycortone, a mineralocorticoid, is inactive. 2-Methylfludrocortisone, which possesses both marked mineralocorticoid and marked glucocorticoid activity, is no more active in producing changes in the amine contents of tissues than other synthetic steroids of similar glucocorticoid potency.

Adrenal cortical steroids are now widely used in the treatment of allergic and inflammatory states. The steroids most effective in these conditions are those which influence the metabolism of carbohydrates. The present results show that the relative effectiveness of the adrenal cortical steroids by injection in allergic states, or by topical application in skin diseases, parallels not only their effectiveness in regulating carbohydrate metabolism but also in reducing the histamine and 5-hydroxytryptamine contents of tissues of the rat such as the skin and small intestine (Table V). The most potent analogues of cortisone have produced depletion of histamine in several tissues which is as great as that produced by the most potent of the chemical histamine liberators. As histamine is one of the mediators of the allergic and inflammatory response, the therapeutic effects of the adrenal cortical steroids in these conditions may be explained in part by their action on histamine metabolism whereby the tissue reserves of this amine are lowered.

The doses of glucocorticoids used clinically in the treatment of allergic diseases are unphysiological, as also are the doses used in the present experiments. Yet the action of any one glucocorticoid in producing depletion of the tissue amines is not always proportional to the dose given. Doses of 0.5 and 1 mg./kg., for example, have been found to produce changes in the histamine and 5-hydroxytryptamine contents of the tissues which are similar to those found after 10 mg./kg., and increasing the dose to 50 mg./kg. (Hicks and West, 1958a) also does not increase the effects.

The tissues chosen for study are all rich sources of histamine and 5-hydroxytryptamine in the rat, but the distribution of the enzymes responsible for the formation and destruction of these amines varies from tissue to tissue. For example, the pyloric stomach is the most potent source of histidine decarboxylase whilst the jejunum is one of the richest sources of histaminase; the skin, however, contains little of either enzyme. Schayer (1956), using tracer techniques, has reported that cortisone increases the histamine-binding capacity of the pyloric stomach, but reduces the histidine decarboxylase activity in the lung and strongly decreases the rate of binding of new histamine in the skin. Recently, Schayer (1960) has postulated that there are at least two types of histidine decarboxylase, one producing histamine which is bound in mast cells and one producing free histamine. Cortisone and the more active adrenal cortical steroids used in the present experiments may influence one of the types preferentially. In fact, the glucocorticoids may stimulate the histidine decarboxylase activity of the pyloric stomach so that the histamine content of this tissue is increased, but reduce the formation of histamine in other tissues.

The effects of adrenalectomy on the histidine decarboxylase and histamine-binding activities of rat tissues are the reverse of those obtained after injections of cortisone (Schayer, 1956). The decrease in the histamine-binding activity of the pyloric stomach, however, does not involve a decrease in histidine decarboxylase activity (Schayer, 1957). Earlier work by Karady, Rose, and Browne (1940) had shown that adrenalectomy in rats results in a loss of histamine activity in the lung and in the small intestine. Thus, the action of adrenal cortical steroids on histamine metabolism appears to involve at least three systems: (1) the formation of histamine as a result of

histidine decarboxylase activity, (2) the binding of histamine with other tissue components, and (3) the inactivation of histamine as a result of histaminase activity. In a similar way, the steroids may modify the formation, binding and inactivation of 5-hydroxytryptamine, since the changes in the tissue levels of this amine are comparable with those of histamine. Studies are now being made on the action of adrenal cortical steroids on the enzymes responsible for the formation and inactivation of both of these amines.

Changes in the appearance of the mast cells occurring during the treatment with glucocorticoids resemble those found when histamine liberators are given. Asboe-Hansen (1952) using cortisone obtained similar results. This author is of the opinion that mast cells are the source of hyaluronic acid in the connective tissue ground substance, and he has suggested that the damaging effect of glucocorticoids on the tissue mast cells may partly explain their clinical value in connective tissue diseases, where the hyaluronic acid content is increased. Several authors (see Asboe-Hansen, 1958) have reported that treatment with corticotrophin likewise reduces the number of mast cells, yet in the present work there were no major changes in the size, shape or number of these cells, nor were there any alterations in the histamine and 5-hydroxytryptamine contents of the tissues. Schayer (1956) also failed to detect any changes in the histidine decarboxylase or histamine-binding activities of various rat tissues after treatment with corticotrophin. The sex hormones, which possess weak adrenal cortical-like activity, similarly did not alter the mast cell population.

Long-term treatment for 9 days with prednisolone, fludrocortisone, and triamcinolone resulted in gastric ulcers in several rats, and also caused petechial haemorrhages in the jejunum and ileum. Histamine is an effective stimulus of gastric secretion of high acidity, and it is thus possible that the increase in the histamine content of the pyloric stomach after treatment with glucocorticoids is the cause of the gastric haemorrhage. Clinically, an important side-effect of glucocorticoid therapy is irritation of the gastric mucosa.

We wish to thank Dr. R. P. Edkins of Organon Laboratories for generous supplies of cortisone acetate and prednisolone. 2-Methylfludrocortisone was kindly supplied by Upjohn of England.

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THE ANATOMICAL BASIS OF THE STRAUB PHENOMENON

BY

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(RECEIVED MAY 31, 1960)

The administration of morphine was followed in white mice by a typical Straub reaction which consisted of the tail becoming rigid and erected across the back of the animal in an S-shaped curve. This reaction was accompanied by restlessness, excitability, extension rigidity of the hindlimbs, forcible viodance of faeces and prominence of the perineum. The Straub reaction was abolished by general anaesthesia with pentobarbitone or ether, by administration of tubocurarine, by bilateral section of the muscles causing extension to the tail, and by the removal of the circulation to the lower extremity. The reaction was modified by unilateral section of the extensor muscles of the tail. Section of the spinal cord, decortication, division of the anal sphincter and perineal floor, or ablation of the pelvic splanchnic nerves did not suppress the appearance of the Straub response. It was concluded that the phenomenon described by Straub (1911) was produced mainly by the action of the sacro-coccygeus dorsalis muscle, and that it was also necessary that the lumbo-sacral cord with its peripheral nervous outflow should be intact and that these functioning units should have an adequate circulation.

Straub (1911) described the sensitive biological reaction for morphine which bears his name. He stated that "when white mice are injected with a small quantity of morphine under the skin of the back, their tails go into a condition of catatonic rigidity which is manifested in such a way that, during the strongest dorsiflexion, the tail is in a position almost parallel to the vertebral column." Subsequent investigators have confirmed and extended these observations. Herrmann (1912) called attention to restlessness, reflex excitability, extension paralysis of the hindquarters, lordosis of the vertebral column and an S-shaped curve of the tail. Van Leersum (1918) reported that defaecation occurred soon after the injection of morphine and that the perineum became prominent. The explanations for the phenomenon found in current textbooks of pharmacology show some disregard for anatomical and functional considerations, particularly in connexion with the skeletal and visceral musculature involved. Gaddum (1955) states that "morphine has a peculiar effect on mice, making them hold their tails erect owing to spasm of the anal and vesical sphincters," and similar explanations are given by Grollman (1958) and Sollman (1957). These explanations appear to stem from the work of Macht (1920), who confused skeletal with smooth muscle activity, and who credited Van Leersum (1918) with showing that "the stiffening of the tail was really due to spasm of the sphincters." The present experiments were carried out in order to investigate the anatomical basis for the Straub and associated reactions which take place in white mice after the administration of morphine.

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METHOD

Thirty-six healthy male and female white mice (T.O. Swiss Strain) of 25 to 30 g. body weight were divided into groups each of 3 animals. Operative procedures where necessary were carried out under sterile surgical conditions using ether anaesthesia. Prior to each observation, the mouse was injected subcutaneously with a solution of morphine hydrochloride at a dose equivalent to 100 mg./kg. of body weight. The observations were continued for a period of 1 hr. following each injection.

RESULTS

Group 1

No treatment and no surgical interference was given to these animals. A typical Straub reaction was always obtained (Fig. 1). Within 30 sec.

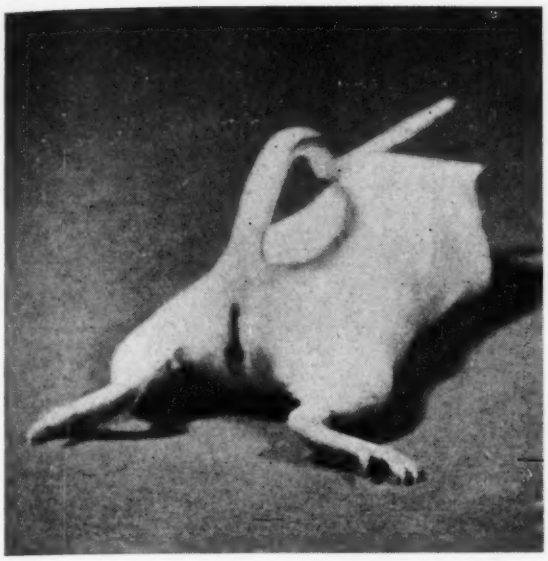

FIG. 1.—The appearance of a white mouse following an injection of morphine. The erection, curvature and disposition of the tail was described by Straub. Other prominent features shown here are the extension paralysis of the hindlimbs, lordosis of the vertebral column, and the prominence of the perineum.

FIG. 2.—Tensing of perineum and voiding of faeces immediately following injection of morphine.

of injection, the animal became restless ; between 2 and 5 min., the tail stiffened and usually took up an S-shaped curvature, and the hindlimbs showed extension rigidity, the animal looking as if it were "stepping on tip-toe." Faeces were voided, the anus protruded slightly, and the perineum became tense (Fig. 2).

Group 2

Before the injection of morphine, each animal was anaesthetized by an intraperitoneal injection of 32 mg./kg. of body weight of sodium pentobarbitone (Nembutal). No Straub reaction occurred under general anaesthesia. If the animal regained consciousness within 10 to 15 min. of the morphine injection, the phenomenon developed. If the period required for the return of consciousness was longer the response was not seen.

Group 3

These animals were anaesthetized with ether before the administration of morphine. No responses were observed while the animals were unconscious. However, a Straub phenomenon gradually appeared as consciousness returned.

Conversely, animals exhibiting the Straub reaction lost the tail rigidity and other features of the phenomenon as soon as deep general anaesthesia was attained.

FIG. 3.—Bilateral cutting of the sacro-coccygeus dorsalis muscle has prevented the erection of the tail in the typical Straub reaction.

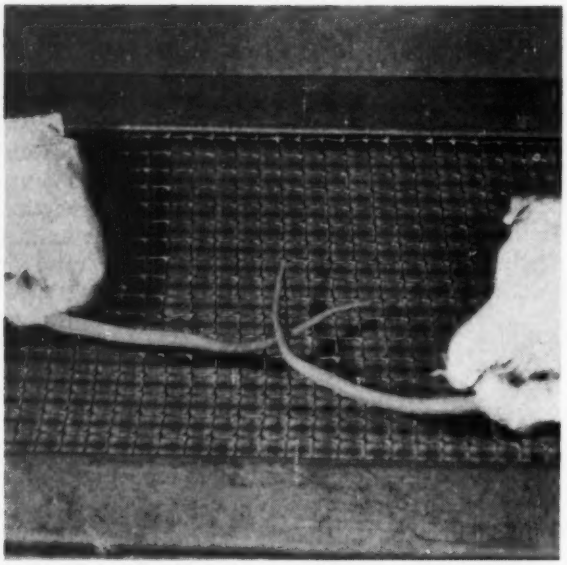

FIG. 4.—After unilateral cutting of the sacro-coccygeus dorsalis muscle, administration of morphine was followed by a deviation of the tail to the side of the intact muscle.

Group 4

Following either subcutaneous or intramuscular injection of tubocurarine (0.0075 mg./animal), the Straub phenomenon could not be elicited. Treatment of an animal showing the Straub reaction with tubocurarine abolished the response.

Group 5

The extensor muscles and tendons to the tail on both sides were sectioned at their attachment to the vertebral column. The stiffening and S-shaped curving of the tail was abolished (Fig. 3), although the other features of the Straub reaction remained.

Group 6

The extensor muscles serving only one side of the tail were sectioned. Stiffening and curving of the tail occurred towards the side of the intact muscles (Fig. 4).

Group 7

The spinal cord was cut across at various levels at the lower thoracic and lumbar segments. Following morphine administration the animal became excited and a moderate stiffening and curling of the tail took place. The hindlimbs which were paralysed went into fuller extension than normal.

Group 8

Decortication did not prevent morphine causing the Straub reaction. The animals became markedly excited and an increased extensor rigidity occurred. Extension paralysis was more prominent than in the intact animal, and was followed by spasms and convulsions.

Group 9

The anal sphincter was divided by cuts along the long axis. Morphine administration was followed by a Straub reaction, but without forcible voidance of faeces or protrusion of the anus.

Group 10

The muscles associated with the perineal floor were cut on both sides of the pelvic outlet. Following morphine, a Straub reaction developed, but there was neither protrusion of the anus nor prominence of the perineum.

Group 11

The pelvic splanchnic nerves were destroyed by the clearance of fascia in the pelvic floor and posterior abdominal wall. When morphine was given, a Straub reaction associated with anal and perineal responses occurred.

Group 12

The abdominal aorta and inferior vena cava were ligated just above the bifurcation of the common iliac vessels. This produced immediate cyanosis of the tail and lower extremities, followed by local paralysis. The Straub reaction could not then be elicited.

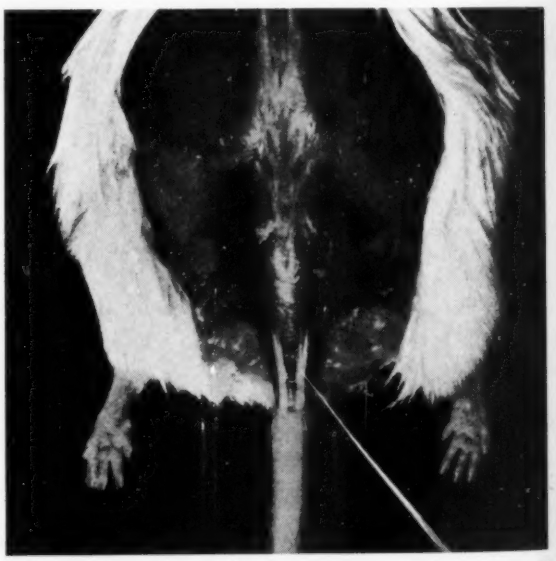

FIG. 5.—Dissection to show the sacro-coccygeus dorsalis muscle which is responsible for elevation and rigidity of the tail. The pointer indicates the tendons leading from this muscle itself which appears as the darkened mass on each side of the vertebral column.

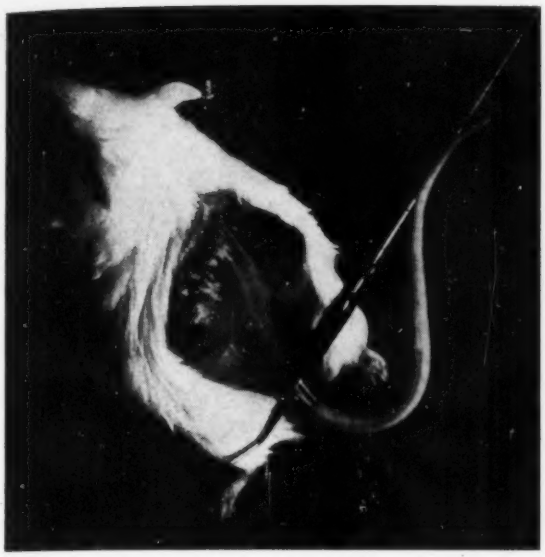

FIG. 6.—Tension on the tendons of the sacro-coccygeus dorsalis muscle has caused the tail to assume a position closely similar to that seen after morphine administration.

DISCUSSION

These experiments provide evidence that the phenomenon described classically by Straub (1911) is dependent upon two primary anatomical units. One neural, composed of segments of the lumbo-sacral cord, the lower motor neurone outflow and functioning motor end-plates. The other musculo-skeletal, consisting of intact musculature to the tail with tendinous attachments to a multi-articulated skeleton.

The muscle specifically responsible for the rigidity, dorsi-extension, and S-shaped curving of the tail was the sacro-coccygeus dorsalis muscle (Fig. 5). Contraction of this muscle gave rise to the typical appearance of the tail following morphine administration (Fig. 6).

Our experiments showed that the constriction of the anal and vesicle sphincters does not appear to be the cause of the Straub phenomenon. However, anal and perineal responses did occur after injection of morphine into white mice, but formed only part of a much wider pattern of muscular activity.

These findings are in agreement with those of Heinekamp (1923), who concluded that "the Straub biologic test is due to direct stimulation of the spinal cord." Furthermore, from electro-encephalographic studies, Leimdorfer (1948) found evidence that the elevation of the tail was a reflex phenomenon originating in the spinal cord. He suggested that it was likely that morphine facilitated an increased activity along the cerebro-spinal pathways.

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BIOLOGICAL ACTIVITY OF SYNTHETIC POLYPEPTIDES WITH BRADYKININ-LIKE PROPERTIES

BY

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The biological activity of synthetic polypeptides containing the amino acids of natural pure trypsin-bradykinin and snake-venom-bradykinin has been investigated. A series of tests for bradykinin-like activity in stimulating plain muscle, depressing the blood pressure and increasing capillary permeability was used on various species. A nonapeptide with the following structure: H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH elicited qualitatively and quantitatively the effects of the pure natural bradykinins. An octapeptide with the following structure: H-L-Arg-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH also exerted bradykinin-like effects but was 50 to 100 times less active than the nonapeptide. Three other octapeptides and a heptapeptide were without any significant effect. Further work will demonstrate if the nonapeptide A is synthetic bradykinin or a peptide with bradykinin-like activity.

Note added since submission of this paper: The data from this investigation were personally precommunicated to Elliott, Lewis, and Horton, who have since found that the structure of pure trypsin-bradykinin is identical with the structure of the nonapeptide A. Therefore, this synthetic nonapeptide is in fact synthetic bradykinin.

Since the discovery of bradykinin, its polypeptide nature has been suggested and amply verified (Rocha e Silva, Beraldo and Rosenfeld, 1949; Rocha e Silva, 1955, 1960). The amino acid composition of bradykinin prepared by the action of trypsin on the fraction of ox plasma precipitating between 35 and 45% of saturation by ammonium sulphate (trypsin-bradykinin) was recently studied by Elliott, Lewis, and Horton (1960a) following its isolation; according to these investigators the molecule of trypsin-bradykinin contains two moles each of arginine, phenylalanine, and proline, and one mole each of glycine and serine. Recently Elliott, Lewis, and Horton (1960b) put forward an octapeptide structure for bradykinin with the amino acid sequence C in Table I.

TABLE I
STRUCTURE OF POLYPEPTIDES A TO F

H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH	A
H-L-Arg-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH	B
H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Phe-L-Arg-OH	C
H-L-Arg-L-Pro-L-Pro-L-Phe-Gly-L-Ser-L-Phe-L-Arg-OH	D
H-L-Arg-L-Pro-Gly-L-Pro-L-Phe-L-Ser-L-Phe-L-Arg-OH	E
H-L-Arg-L-Pro-Gly-L-Phe-L-Ser-L-Phe-L-Arg-OH	F

On this basis, Boissonnas, Guttmann, and Jaquenoud (1960a, b) have synthesized this octapeptide and five other related polypeptides (Table I). Whereas the polypeptide having the structure proposed for bradykinin (C in Table I) showed practically no bradykinin-like activity, another octapeptide with the same amino acids but in a different sequence (B in Table I) was active (Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer, 1960). Still more activity was exhibited by a nonapeptide: H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH containing three proline residues instead of two (A of Table I, Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer, 1960; Boissonnas, Guttman, and Jaquenoud, 1960a).

The investigations described below are mainly concerned with a description of the biological activity of this potent nonapeptide and other related peptides synthesized by Boissonnas, Guttmann, and Jaquenoud (1960a, b). A brief description of some of the findings has already been

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published (Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer, 1960).

The new peptides were tested on a number of biological preparations known to be sensitive to bradykinin (Rocha e Silva, 1952, 1955, 1960; Holdstock, Mathias, and Schachter, 1957; Hamberg, 1959; Collier, Holgate, Schachter, and Shorley, 1959; Elliott, Horton, and Lewis, 1960).

METHODS

Materials.—The polypeptides A to F (as hydrochlorides) in an analytically pure state were kept in stock solutions in water containing 1 mg. or 0.1 mg. of free peptide per ml. in a refrigerator at +4°. Saline was used for further dilutions.

In some experiments a comparison was made with a preparation of natural trypsin-bradykinin prepared by Dr. J. F. Pechère from bovine plasma by incubation with trypsin and partly purified by a modification of the method of Hamberg and Deutsch (1958); in other experiments acetylcholine chloride, histamine dihydrochloride, adrenaline hydrochloride, and pure synthetic oxytocin were used for comparison. Atropine sulphate and thenalidine tartrate were sometimes employed to differentiate effects due to bradykinin from those elicited by acetylcholine and histamine. The doses refer to the salts.

Isolated Smooth Muscles.—Preparations of ileum and seminal vesicle of the guinea-pig and of rabbit duodenum were suspended in Tyrode solution at 37°.

Preparations of rat uterus were suspended in de Jalon solution at 30°. On the day preceding the experiment the rats were treated with 0.2 mg. stilboestrol subcutaneously.

The organ baths for the guinea-pig ileum and the rat uterus contained 10 ml. solution; those for the other organs contained 50 ml.

Smooth Muscles in vivo.—To record bronchoconstrictor action the overflow method of Konzett and Rössler (1940) with positive-pressure ventilation was used on guinea-pigs anaesthetized with urethane (1.2 g./kg. intraperitoneally) and on spinal cats.

Arterial Blood Pressure.—For anaesthesia a mixture of chloralose (0.05 g./kg.) and urethane (0.4 g./kg.) was given subcutaneously in cats and intravenously in dogs; rabbits received 1.6 g./kg. urethane subcutaneously; rats were injected with 2.0 g./kg. urethane subcutaneously; guinea-pigs were given 1.2 g./kg. urethane intraperitoneally; roosters were anaesthetized with 0.2 g./kg. phenobarbitone sodium intramuscularly.

Injections were made into the femoral, jugular, or brachial vein; arterial blood pressure was recorded from a carotid, femoral, or sciatic artery by means of a mercury manometer.

In some experiments on the cats the renal volume (left kidney) was recorded plethysmographically by means of a Roy oncometer.

In some cats and dogs the respiration was recorded concomitantly with the blood pressure either by means of a Gildemeister (1922) apparatus (gas meter) or simply by a rubber cuff lying around the lower thorax and connected to a Marey tambour.

Capillary Permeability.—Guinea-pigs were given 0.2 ml. of a 2% solution of Evans blue into the saphenous vein; 1 hr. later, intradermal injections of the test substances (0.1 ml.) were made on the back at positions approximately 10 mm. from the midline. To estimate the effect of various substances and doses, the animals were killed and depilated 1 hr. after injection, and a comparison was made of the intensity of the blue colouring around the injection site.

RESULTS

Effects on Smooth Muscle

In vitro *Preparations.*—The nonapeptide A produced a delayed slow contraction of all smooth muscle structures so far investigated. The *guinea-pig ileum* seemed the most suitable for an assay, since it was fairly sensitive and graded responses were available over a reasonable range of concentrations. Nonapeptide A was effective when given in doses of 1 ng./ml. or more. Fig. 1 shows the slow contraction of the guinea-pig ileum elicited by nonapeptide A as compared with the quick contraction produced by histamine

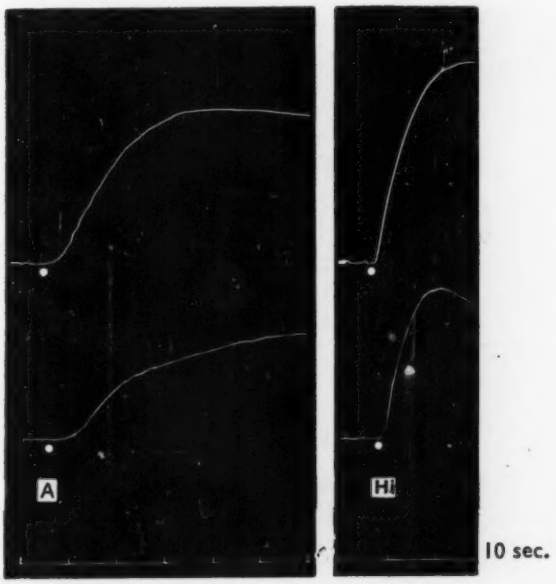

FIG. 1.—Isolated guinea-pig ileum; 10 ml. bath. Responses of two pieces of ileum to 16 ng./ml. of nonapeptide A (A) and histamine (Hi); drum speed 78 mm./min. Note the difference between the delayed contractions after nonapeptide A and the quick contractions after histamine. Time: 10 sec.

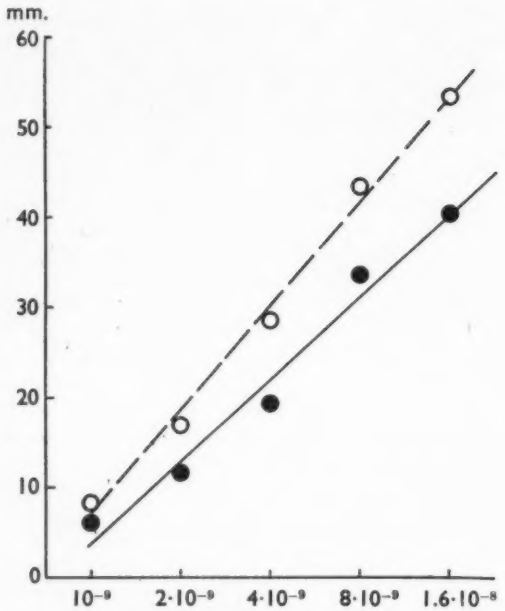

FIG. 2.—Dose-response curve for the nonapeptide A ●—● and histamine O—O on the isolated guinea-pig ileum. Each point represents the mean of 13 to 21 experiments.

(submaximal doses of both compounds). Dose-response curves of nonapeptide A and (for comparison) of histamine under the same conditions are depicted in Fig. 2. The curves lie close together, and their slope differs only slightly. In both instances there is a straight-line relationship between the log dose and the response. Nonapeptide A was nearly as active as histamine (weight for weight).

On the guinea-pig ileum, octapeptide B gave the same type of response as nonapeptide A, but was about 100 times less active.

The peptides C, D, E in doses up to 10 μg./ml. and the peptide F up to 2.5 μg./ml. were without effect on the guinea-pig ileum.

Atropine 10 ng./ml. and an antihistamine (thenalidine) 20 ng./ml. added to the bath for 5 min. completely abolished the effects of submaximal doses of acetylcholine or histamine respectively and reduced the effect of the peptides A and B by about 40%. The same observation was made with the partly purified bradykinin preparation. These inhibitory effects of atropine and of the antihistamine are not specific, since a reduction of the same magnitude was also seen when histamine was given after atropine or acetylcholine was added after the antihistamine.

However, the effect of the peptides A and B was completely abolished when they were boiled with concentrated hydrochloric acid. Also, incubation with undiluted guinea-pig serum 0.6 μg. of A or 60 μg. of B in 0.5 ml. saline + 0.1 ml. serum for 30 sec. for A and 15 min. for B abolished the effects of these peptides on the guinea-pig ileum.

The *duodenum of the rabbit* was also sensitive to nonapeptide A, although the response varied from preparation to preparation to a greater extent than in the case of the guinea-pig ileum. Nonapeptide A was effective in doses of 1 ng./ml. Fig. 3 shows—following an initial relaxation—the slow contraction after nonapeptide A and the quick contraction after acetylcholine. Small

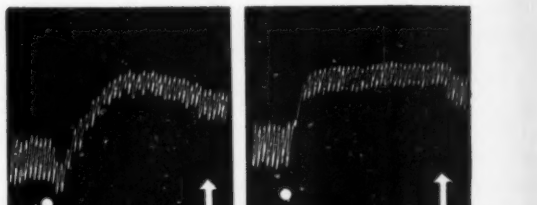

FIG. 3.—Isolated rabbit duodenum; 50 ml. bath. Responses to 2.5 ng./ml. nonapeptide A (A) and to 5 ng./ml. acetylcholine (Ach). Drum speed 14 mm./min. At the arrows: washing out. Note the slow contraction after nonapeptide A and the quick contraction after acetylcholine. Time: 1 min.

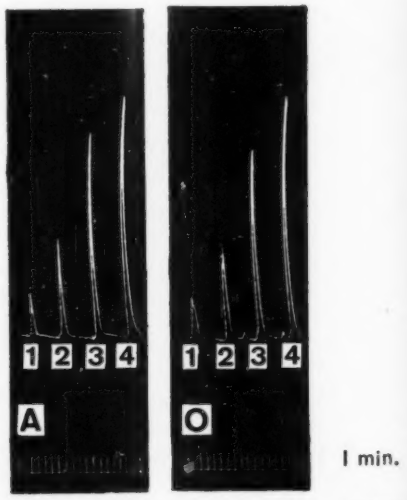

FIG. 4.—Isolated rat uterus; 10 ml. bath. Contractions after increasing doses of nonapeptide A (A) and pure synthetic oxytocin (O). Doses of A: 1= 0.1 ng./ml.; 2=0.13 ng./ml.; 3=0.2 ng./ml.; 4= 0.33 ng./ml. Doses of O: 1=0.044 ng./ml.; 2= 0.066 ng./ml.; 3=0.088 ng./ml.; 4=0.11 ng./ml. Time: 1 min.

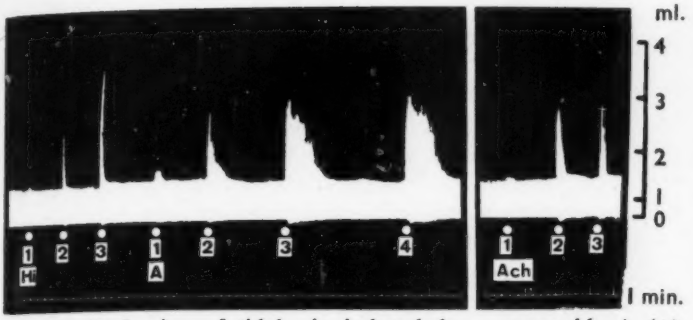

FIG. 5.—Reduction of tidal air induced by nonapeptide A (A), histamine (Hi), and acetylcholine (Ach) in the guinea-pig (0.5 kg.). Doses of Hi: 1=2 μg./kg.; 2=4 μg./kg.; 3=8 μg./kg. Doses of A: 1=0.1 μg./kg.; 2=0.2 μg./kg.; 3=0.4 μg./kg.; 4=0.8 μg./kg. Doses of Ach: 1=10 μg./kg.; 2=20 μg./kg.; 3=40 μg./kg. Time: 1 min.

doses of both compounds were used. Owing to the spontaneous pendulum movements, quantitative determination is not as easy on the rabbit duodenum as on the guinea-pig ileum. A bracketing procedure in which nonapeptide A and acetylcholine were given alternately to pieces of the duodenum showed that the activity of nonapeptide A was similar to that of acetylcholine (weight for weight): sometimes it was more active and sometimes it was less active. No exact quantitative comparison of both compounds was made.

The most sensitive preparation so far known is the *rat uterus*, which responded to doses of 0.03 ng./ml. nonapeptide A. However, the dose-response relationship on the rat uterus is occasionally not as good over a wide range of doses as is that on the guinea-pig ileum. In Fig. 4 the effects of increasing doses of nonapeptide A and of oxytocin are shown. Oxytocin was, on a weight basis, usually more active than nonapeptide A (Fig. 4). Dose-response curves of nonapeptide A and of oxytocin showed a straight-line relationship between the log dose and the response. However, the line belonging to oxytocin is steeper.

Octapeptide B also elicited contractions of the rat uterus. Comparable effects were observed after doses which were 70 times greater than those of nonapeptide A.

The peptides C, D, and E were without effect on the rat uterus in doses up to 10 μg./ml. The peptide F was not tested on the rat uterus.

The preparation least sensitive to nonapeptide A was the *seminal vesicle of the guinea-pig*. 0.5 μg./ml. was needed to elicit a contraction. In this respect nonapeptide A was about as active as adrenaline (weight for weight).

In vivo *Preparations*.—Nonapeptide A produced a decrease in tidal air in anaesthetized guinea-pigs (6 experiments) and spinal cats (4 experiments). This effect on the distensibility of the lungs is in all probability due to bronchoconstriction (Konzett, 1956).

The guinea-pig lungs were rather sensitive; an effect was noted with doses as small as 0.2 μg./kg. (see Fig. 5). Increasing the dose of nonapeptide A enhanced either the duration or the degree of the effect.

Under comparable conditions histamine was (weight for weight) approximately 20 times less active than nonapeptide A and acetylcholine was 100 times less active (Fig. 5). In contrast to nonapeptide A, even high doses of histamine and acetylcholine did not produce a long-lasting effect.

Octapeptide B was also active in the guinea-pig preparation. The doses needed were about 50 times greater than that of nonapeptide A.

In spinal cats the doses of nonapeptide A required to decrease the tidal air were higher than these in guinea-pigs. Less than 10 μg./kg. was usually inactive. Fig. 6 shows the effect of increasing doses (12.8 to 102.4 μg./kg.) of nonapeptide A in a sensitive preparation. The effect of 1 μg./kg. histamine is also shown to illustrate

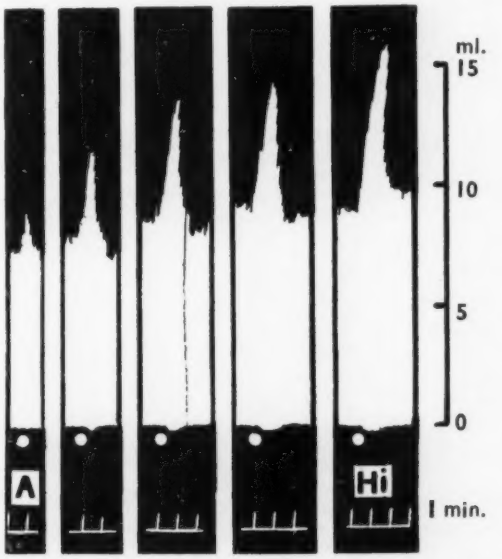

FIG. 6.—Reduction of tidal air induced by nonapeptide A (A) and histamine (Hi) in the spinal cat (2.6 kg.). Doses of A (from left to right): 12.8 μg./kg., 25.6 μg./kg., 51.2 μg./kg., and 102.4 μg./kg.; dose of histamine: 1 μg./kg. Time: 1 min.

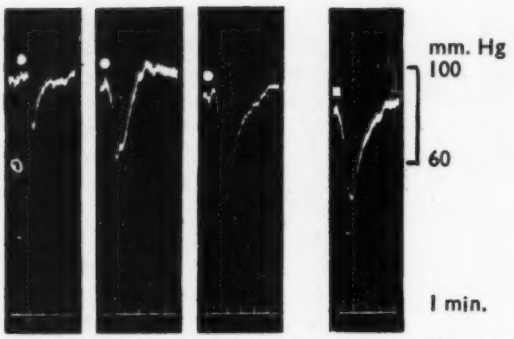

FIG. 7.—Effect of nonapeptide A (●) and of acetylcholine (■) on the blood pressure of the rabbit (3.7 kg.). Doses of nonapeptide A (from left to right): 0.05 μg./kg., 0.1 μg./kg., and 0.2 μg./kg.; dose of acetylcholine: 0.2 μg./kg. Time: 1 min.

the different sensitivity of the bronchial muscle of the spinal cat to the two compounds. In contrast to the guinea-pig, histamine was here more active (approximately 100 times) than nonapeptide A.

Effects on the Arterial Blood Pressure of Different Species

Nonapeptide A lowered the arterial blood pressure of all mammals so far investigated, namely, cat (13 experiments), dog (6 experiments), rabbit (4 experiments), rat (4 experiments), and guinea-pig (6 experiments). In higher doses it even reduced the blood pressure of the chicken (4 experiments).

A comparison of the smallest doses needed to elicit a definite fall in blood pressure in the different species showed the rabbit to be the most sensitive animal towards nonapeptide A (see Fig. 7); 0.05 μg./kg. elicited a barely detectable fall in blood pressure (in 3 of 4 experiments). In the dog, rat, and guinea-pig blood pressure fell after doses around 0.2 μg./kg.; and in the cat (spinal and anaesthetized) doses of 0.5 μg./kg. and more were usually needed.

The depressor effect of threshold doses of nonapeptide A was very short-lasting. Increasing the doses produced greater responses of longer duration. When given at intervals of 15 min. or more, the depressor action of small and medium doses could easily be reproduced in all species.

Fig. 7 gives an example of the blood pressure response to increasing doses of nonapeptide A in the rabbit. The effect of acetylcholine is shown for comparison. In the rabbit (Fig. 7) acetylcholine (on a weight for weight basis) exerts a weaker depressor effect than nonapeptide A.

In dogs, guinea-pigs, and rats there was also a good dose-response relationship between log-dose of nonapeptide A and the fall of blood pressure. Nonapeptide A was more active than acetylcholine on a weight for weight basis in dogs (4 out of 5 experiments) and more active than histamine (6 experiments) in the guinea-pig.

In the anaesthetized cat the effect of relatively high doses of nonapeptide A is shown in Fig. 8. Initially renal volume closely follows the change in blood pressure, but after large doses it increases before the blood pressure has reached the original level. The increase in renal volume seems to

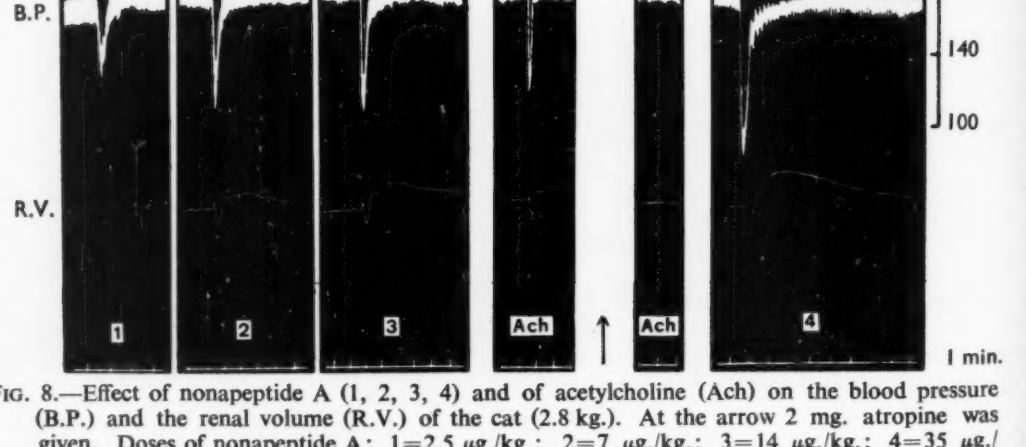

FIG. 8.—Effect of nonapeptide A (1, 2, 3, 4) and of acetylcholine (Ach) on the blood pressure (B.P.) and the renal volume (R.V.) of the cat (2.8 kg.). At the arrow 2 mg. atropine was given. Doses of nonapeptide A: 1=2.5 μg./kg.; 2=7 μg./kg.; 3=14 μg./kg.; 4=35 μg./kg.; dose of acetylcholine: Ach=0.23 μg./kg. Time: 1 min. Note: Atropine completely abolished the effect of acetylcholine but did not interfere with that of nonapeptide A.

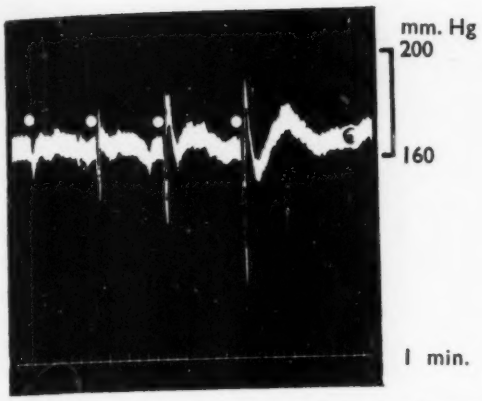

FIG. 9.—Effect of nonapeptide A on the blood pressure of the rooster (1.3 kg.). Doses (from left to right): 37 μg./kg., 74 μg./kg., 148 μg./kg., 296 μg./kg. Time: 1 min.

depend on the dose of nonapeptide A. Fig. 8 also illustrates that atropine does not interfere with the depressor action of nonapeptide A but abolishes the effect of acetylcholine. In the dog also atropine does not influence the depressor effect of nonapeptide A.

During the fall of blood pressure elicited by small and medium doses (0.4 to 3.0 μg./kg.) of nonapeptide A the heart rate rose in the dog but remained nearly unchanged in the rabbit and the cat. At the same time, respiratory rate and volume increased in the dog but not in the cat.

The highest single doses of nonapeptide A given in the course of an experiment were 12.8 μg./kg. in the dog and 300 μg./kg. in the cat. They were not lethal.

In the chicken much higher doses were required to produce a fall of blood pressure. The threshold doses in the rooster were about 40 to 80 μg./kg. (see Fig. 9). There was a fairly good dose-response relationship. Comparable depressor effects on the chicken blood pressure were achieved with approximately 1,000 times smaller doses of acetylcholine.

The fall of blood pressure in the rooster was sometimes followed by a secondary rise to above the original level (see Fig. 9); both the initial fall and the secondary rise of blood pressure were of short duration.

Effects on Capillary Permeability

After intradermal injection nonapeptide A increased the capillary permeability in the skin of guinea-pigs pretreated with Evans blue in 23 experiments (Fig. 10). Doses as small as 1 ng./

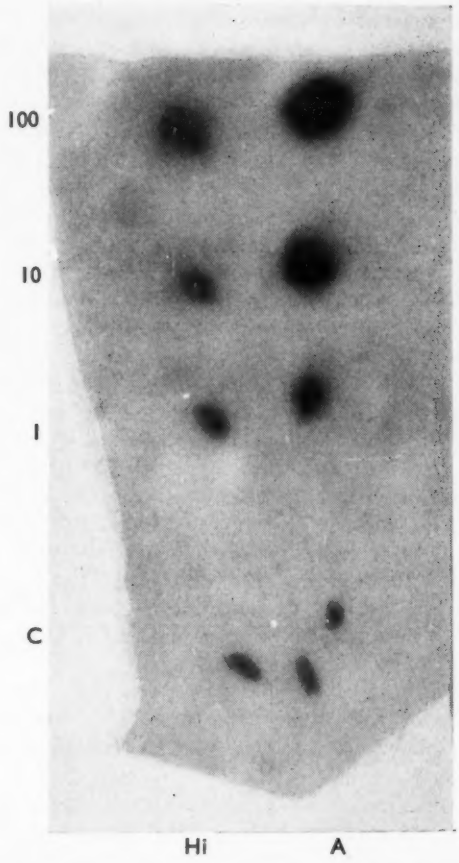

FIG. 10.—Effect of intradermal injections of nonapeptide A and histamine in increasing capillary permeability to circulating Evans blue in the guinea-pig. Dye accumulates at intradermal injection sites. A, nonapeptide A; Hi, histamine; C, control injections with the same volume of saline. Numerals represent the injected doses in ng./0.1 ml. Photo by transmitted light. Note: A comparison of identical doses shows that the extent and density of the "blue" areas are greater after nonapeptide A than after histamine.

0.1 ml. had a definite effect (13 of 16 experiments). The action of histamine under the same conditions in the same animals was about 10 times weaker than that of nonapeptide A.

DISCUSSION

The above-mentioned experiments demonstrate that nonapeptide A (Table I) exhibits actions of bradykinin from natural sources prepared either by the action of trypsin (=trypsin-bradykinin) or by the action of snake-venom (=snake-venom-bradykinin). These actions—slow contraction of the smooth muscle, depression of arterial blood

pressure, and increase in capillary permeability—are inherent in this synthetic nonapeptide A as well as in trypsin-bradykinin and snake-venom-bradykinin. Therefore, the *qualitative* comparison so far shows no discrepancy between the effect of natural bradykinin and that of the synthetic nonapeptide A.

Bradykinin and nonapeptide A react in an identical manner to various procedures. Boiling with concentrated hydrochloric acid destroys snake-venom-bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949) and nonapeptide A; incubation with guinea-pig serum abolishes the action of trypsin-bradykinin (Rocha e Silva, 1955) and of nonapeptide A. Antihistamines and/or atropine exhibit no specific antagonism to snake-venom-bradykinin and trypsin-bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949) and to nonapeptide A. The enzyme chymotrypsin splits arginine from pure trypsin-bradykinin (Elliott, Lewis, and Horton, 1960b) and from nonapeptide A (Boissonnas, Guttmann, and Jaquenoud, 1960a). Further enzymatic treatment by carboxypeptidase splits phenylalanine from the residue of trypsin-bradykinin (Elliott, Lewis, and Horton, 1960b) and from the octapeptide formed from nonapeptide A (Boissonnas, Guttmann, and Jaquenoud, 1960a).

There are also *quantitative* data indicative of a close similarity between bradykinin and nonapeptide A. The rat uterus is 25 to 30 times more sensitive to bradykinin than the guinea-pig ileum (Andrade and Rocha e Silva, 1956). The same relationship was observed for the sensitivity of both organs towards nonapeptide A. Elliott, Horton, and Lewis (1960) found 0.2 ng./ml. of pure natural trypsin-bradykinin was required to contract the rat uterus; this accords with the dose of 0.1 ng./ml. of the synthetic nonapeptide necessary to contract the uterus. The dose of 400 ng./kg. of pure natural trypsin-bradykinin required to produce a depressor response in cats which is mentioned by Elliott, Horton, and Lewis (1960) is in close agreement with the smallest dose of approximately 500 ng./kg. of the synthetic nonapeptide A needed to elicit a blood pressure fall in cats. And finally, the threshold dose of pure natural snake-venom-bradykinin (1 ng./ml.) for contraction of the isolated guinea-pig ileum (Zuber and Jaques, 1960) is identical with the threshold dose of nonapeptide A in this organ.

All these qualitative criteria and the quantitative data available for pure bradykinins reveal a very close similarity between nonapeptide A and natural bradykinin.

As far as we can see there is only one discrepancy between a property of purified snake-venom-bradykinin that has been reported and the action of nonapeptide A, namely, the effect on the blood pressure of the fowl. Rocha e Silva (1960) states that bradykinin was entirely without effect on the blood pressure of the fowl. This is based on experiments in which not pure bradykinin but only partially purified bradykinin was used. On giving high doses of nonapeptide A we observed a definite fall of the arterial blood pressure of the rooster (see Fig. 9); but on a weight basis nonapeptide A was more than 10,000 times less active than oxytocin. The doses of bradykinin required to produce a fall in blood pressure of the chicken were approximately 100 to 200 times higher than the doses required for the same effect in mammals. Rocha e Silva probably did not give sufficiently high doses of partly purified bradykinin to the fowl to elicit a blood pressure fall. His statement (1960) that oxytocin is without effect on the mammalian pressure also does not accord with other observations (du Vigneaud, 1955; Van Dyke, Adamsons and Engel, 1955; Gyermek and Fekete, 1955; Konzett, Berde, and Cerletti, 1956), possibly for the same reason. Therefore, the discrepancy between the reported failure to produce a blood pressure fall with bradykinin in the fowl and our observation that nonapeptide A in very high doses is also depressor in the chicken does not necessarily constitute a difference.

Taking all the above-mentioned facts into consideration we come to the conclusion that nonapeptide A is identical with or at least very closely related to bradykinin.

To give an idea of the general biological activity of nonapeptide A, a comparison may be made with the very potent naturally occurring amines histamine and acetylcholine. Weight for weight, nonapeptide A is more active than histamine in reducing tidal air in guinea-pigs and in causing a fall of blood pressure on the rabbit, guinea-pig, and often on the dog. On a molar basis nonapeptide A is also more active than histamine on the guinea-pig ileum and more active than acetylcholine on the rabbit duodenum.

A comparison of nonapeptide A with oxytocin shows that on the isolated rat uterus nonapeptide A is only 1.5 to 3 times less active than oxytocin (weight for weight and on a molar basis). On the blood pressure of the chicken, however, nonapeptide A is more than 10,000 times less active than oxytocin (weight for weight and on a molar basis).

The synthetic octapeptide (C in Table I) with the sequence of amino acids reported by Elliott, Lewis, and Horton (1960b) to occur in trypsin-bradykinin is without effect even in high concentrations (10 μg./ml. on the guinea-pig ileum and the rat uterus). This is confirmed by a preliminary report of Schwyzer, Rittel, Sieber, Kappeler, and Zuber (1960), who also synthesized this octapeptide and found it to be without effect on the isolated guinea-pig ileum in doses up to 10 μg./ml.

It is, however, remarkable that recently after isolation of a bradykinin resulting from the action of snake-venom on bovine plasma, Zuber and Jaques (1960) have stated that this snake-venom-bradykinin in an essentially pure form contains the five amino acids arginine, phenylalanine, proline, glycine, and serine in the ratio of 2:2:2:1:1. It therefore corresponds to the amino acid composition found for trypsin-bradykinin by Elliott, Lewis, and Horton (1960a). As yet, Zuber and Jaques (1960) have not published the whole sequence of the amino acids.

The fact that an octapeptide with these above-mentioned amino acids can exhibit bradykinin-like properties on the isolated guinea-pig ileum, on the rat uterus, and on the tidal air of guinea-pigs is demonstrated in the example of the synthetic octapeptide B (in Table I). As in the case of bradykinin and of nonapeptide A the rat uterus was more sensitive (approximately 20 times) than the guinea-pig ileum to octapeptide B. Boiling with concentrated hydrochloric acid or incubating with guinea-pig serum abolishes the action of octapeptide B on the isolated guinea-pig ileum. This octapeptide B, however, is markedly less potent (50 to 100 times) in various tests (isolated guinea-pig ileum, isolated rat uterus, tidal air of anaesthetized guinea-pigs) than pure trypsin-bradykinin (Elliott, Horton, and Lewis, 1960) and pure snake-venom-bradykinin (Zuber and Jaques, 1960). It is thus definitely not identical with them.

The other peptides synthesized which contain the above-mentioned amino acids, namely, the octapeptides D and E (Table I) and the heptapeptide F, were without effect on the isolated guinea-pig ileum even in high doses (10 μg./ml. for D and E and 2.5 μg./ml. for F). The octapeptides D and E had in the same doses also no effect on the isolated rat uterus, whereas the heptapeptide F was not investigated on this organ.

The above-mentioned results with the various peptides containing the amino acids of natural bradykinin necessitate a re-examination of the structure of natural bradykinin. The present somewhat complicated situation in the bradykinin field may then be clarified.

Addendum after submission of this paper: In view of the previously reported biological actions of the synthetic nonapeptide A, Elliott, Lewis, and Horton (1960c) have reinvestigated the structure of natural trypsin-bradykinin, and, after new degradation studies, have presented evidence that natural bradykinin is a nonapeptide having the structure of nonapeptide A. Therefore this synthetic nonapeptide is in fact synthetic bradykinin.

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A STUDY OF POTENTIAL HISTIDINE DECARBOXYLASE INHIBITORS

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A series of compounds has been examined for ability to inhibit histidine decarboxylase. Histidine analogues having substituents in the imidazole ring showed a wide variation in potency, but these were all much less active than α-methyldopa [β-(3,4-dihydroxyphenyl)-α-methylalanine], the most potent known inhibitor of histidine decarboxylase. Some tentative conclusions are drawn regarding the relationship between chemical structure and inhibitory activity.

Numerous substances have been tested for their ability to inhibit the enzyme histidine decarboxylase (Werle and Heitzer, 1938; Werle, 1940, 1942; Martin, Graff, Brendel, and Beiler, 1949; Werle and Koch, 1949; Werle, Schauer, and Hartung, 1955; Waton, 1956; Udenfriend, Lovenberg, and Weissbach, 1960). Although several potent inhibitors were found in the course of these studies, none was sufficiently specific to inhibit histidine decarboxylase without simultaneously inhibiting other enzymes. In particular, compounds which act by influencing the availability of the co-decarboxylase pyridoxal 5′-phosphate will inhibit not only histidine decarboxylase but all amino-acid decarboxylases and the transaminases, for which pyridoxal phosphate is also the co-enzyme.

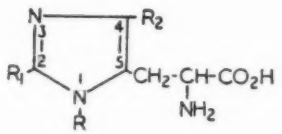
A series of compounds, some of them structural analogues of histidine, has been studied in an attempt to clarify the nature of the active centre of the enzyme, and to determine whether they can inhibit histidine decarboxylase specifically by competition with the normal substrate.

A specific, competitive inhibitor of histidine decarboxylase would be useful as a pharmacological tool. In view of the recent work of Kahlson and co-workers (see Kahlson, 1960) and by Riley and co-workers (Mackay, Marshall, and Riley, 1960), which indicates that histidine decarboxylase may play some part in normal and abnormal growth processes, such a compound might have therapeutic applications.

METHODS

Synthesis of Compounds

Histidine Analogues.—These corresponded to the general formula I. Where substituents could not be introduced directly into the imidazole ring of histidine, the method of Jones and McLaughlin (1949) was employed, the starting material in each case being the correspondingly substituted hydroxymethylimidazole.



I

The position of the iodine atom in L-monoiodohistidine is unknown (Brunings, 1947).

In naming the imidazole derivatives it should be noted that, except in cases where one of the nitrogen atoms in the imidazole ring carries a substituent, positions 4 and 5 are equivalent owing to tautomerism.

5-Hydroxymethyl-2-methylimidazole Hydrochloride.—This was prepared by reacting dihydroxyacetone, acetaldehyde, ammonia, and basic copper carbonate, under the conditions described by Totter and Darby (1944) for the preparation of 5-hydroxymethylimidazole hydrochloride. The compound was isolated as the picrate, and converted to the hydrochloride in the usual way.

4-Hydroxymethyl-1-methylimidazole Hydrochloride.—This was prepared as the picrate by the method of Ruoff and Scott (1950), and converted to the hydrochloride.

In Table I are given the melting points and analyses of new compounds prepared in the course of these studies. The following compounds were prepared as described in the literature: 4-hydroxymethyl-1-methylimidazole picrate (Ruoff and Scott, 1950); 4-methylimidazole (Bernhauer, 1929); 5-hydroxymethyl-4-methylimidazole hydrochloride (Ewins, 1911); and 4-methyl-5-nitroimidazole (Allsebrook, Gulland, and

TABLE I

PROPERTIES OF COMPOUNDS PREPARED. ALL AMINO-ACIDS ARE DL-FORMS

Compound	Molecular Formula	Analyses								m.p.°
		Calculated %				Found %				
		C	H	N	Cl	C	H	N	Cl	
5-Hydroxymethyl-2-methyl-imidazole picrate ..										172–3
5-Hydroxymethyl-2-methyl-imidazole hydrochloride	$C_5H_9ON_2Cl$	40·6	6·1	18·9	—	40·4	5·7	18·8	—	151–2
5-Chloromethyl-2-methyl-imidazole hydrochloride	$C_5H_8N_2Cl_2$	—	—	—	42·2	—	—	—	41·4	149–150
β-(2-Methylimidazol-5-yl)-alanine dihydrochloride (2-methylhistidine) ..	$C_7H_{13}O_2N_3Cl_2$	34·9	5·3	17·4	—	34·6	5·5	16·8	—	257–9 (dec.)
4-Hydroxymethyl-1-methyl-imidazole hydrochloride	$C_5H_9ON_2Cl$	40·6	6·1	18·9	—	39·9	6·1	19·6	—	124–5
4-Chloromethyl-1-methyl-imidazole hydrochloride	$C_5H_8N_2Cl_2$									153–4
β-(1-Methylimidazol-4-yl)-alanine dihydrochloride	$C_7H_{13}O_2N_3Cl_2$	34·9	5·3	17·4	—	35·3	5·6	17·6	—	251–3 (dec.)
β-(4-Methylimidazol-5-yl)-alanine monohydrochloride monohydrate (4-methyl-histidine)	$C_7H_{14}O_3N_3Cl$	37·7	6·3	18·8	15·7	38·5	6·5	17·5	15·1	168–170 (dec.)

Story, 1942). Where melting points were in agreement with those already published, analyses were not carried out.

Histidine.—It was necessary to prepare L-histidine zwitterion from commercial L-histidine monohydrochloride monohydrate to eliminate traces of histamine present. The hydrochloric acid was removed by adding excess silver carbonate to a warm concentrated aqueous solution of the monohydrochloride. After filtration, silver ions were removed from the filtrate by passing in hydrogen sulphide. Filtration and subsequent concentration of the filtrate, under reduced pressure, gave a saturated solution of L-histidine zwitterion from which the latter was precipitated by addition of dehydrated alcohol.

Enzyme Studies

Preparation of Histidine Decarboxylase.—Guinea-pig kidneys were used as the source of mammalian histidine decarboxylase. They were pulped with purified sand, approximately 30 ml. of saline was then added/g. of tissue, and the mixture was ground once more. The fluid was centrifuged at 15,000 *g* for 20 min., and the supernatant was used as a crude enzyme extract.

Incubation Experiments.—The incubation medium for the uninhibited reaction was prepared by pipetting into a centrifuge tube phosphate buffer of *p*H 8.0 (isotonic with blood), 2.0 ml.; 0.9% sodium chloride solution, 0.9 ml.; L-histidine zwitterion solution (25.0 mg./ml. saline), 1.0 ml.; and 3×10^{-3} M aminoguanidine bicarbonate in saline, 0.1 ml. After the tubes and their contents had been left in the thermostat for about 15 min., 1.0 ml. of enzyme extract was added, the tubes were stoppered, inverted to mix the solutions, and the time was noted. The final volume in each tube was 5.0 ml. so that the final concentrations of L-histidine and aminoguanidine were respectively 32.2×10^{-3} M and 6×10^{-3} M. Aminoguanidine in the concentration used has been shown by Waton (1956) to inhibit completely any histaminase present; it was added as a routine precaution, though the histaminase activity of guinea-pig kidney extracts is very low. In order to measure the rate of the reaction in presence of decarboxylase inhibitor, the latter was added either in solid form or as a neutralized solution in saline, with the volume of pure saline added to the incubation medium correspondingly reduced. Inhibition was therefore studied at constant *p*H, temperature and substrate concentration. Since the enzyme concentration was constant only for a given extract at a given time, simultaneous studies on the inhibited and uninhibited reaction had to be carried out using the same extract.

The reaction was stopped by adding 0.20 ml. of 2 N hydrochloric acid to the centrifuge tubes to make the media just acid, and boiling for 1 min. to coagulate protein. The latter was centrifuged down, and the

solutions were stored overnight at 4°. The histamine produced was estimated by the 4-point method on atropinized guinea-pig ileum at 34°, using an automatic assay apparatus. Control experiments were carried out to detect any histamine either in the reagents, or produced by interaction of enzyme with the inhibitors. The effect of inhibitors on the assay of histamine was checked, and the stability of histamine under the conditions used was also studied. No serious complications were encountered.

Estimation of Inhibitor Potency.—An approximate test of the inhibitor potency was made by simply incubating the inhibitor with L-histidine and enzyme extract at 36° for about 3 hr. Then an approximate estimate was made of the molar concentration of inhibitor required to reduce the velocity of the uninhibited reaction by 50% (C_{50}). Two concentrations of inhibitor, calculated to produce about 30% and 70% inhibition, were then chosen for more detailed study. The incubation media were prepared as described, the volumes in each tube being increased to 25 ml., so that samples (7 ml.) could be removed at known times after the addition of the enzyme. The samples were made just acid, boiled, centrifuged, and stored at 4° until assayed.

Results

Graphs were prepared in which the concentration of histamine in the incubation mixtures was plotted against time. From each graph the rate of production of histamine at the beginning of the reaction (initial velocity) was determined.

The variation of the initial velocity with substrate concentration, at constant enzyme concentration, was determined in a preliminary experiment. The results (Fig. 1) show that the substrate concentration used was well below the level which would saturate the enzyme.

From a plot of the reciprocal of the initial velocity against the concentration of inhibitor in the medium the C_{50} value was obtained as shown in Fig. 2, which represents an ideal case. This inhibitor concentration is related to the inhibitor-enzyme equilibrium constant, the exact relationship depending on whether the inhibition is competitive or non-competitive (Lineweaver and Burk, 1934). When the type of inhibition is not known with certainty, as in the present examples, the C_{50} value can still be used as an index of the inhibitory power of the compound under test. The reproducibility of the C_{50} determination was tested with D-histidine using 3 different guinea-pig extracts; the error in C_{50} obtained in this way may be as much as 20%. The C_{50} values for the compounds tested are given in Table II. It should be noted that they depend on the substrate concentration and *p*H.

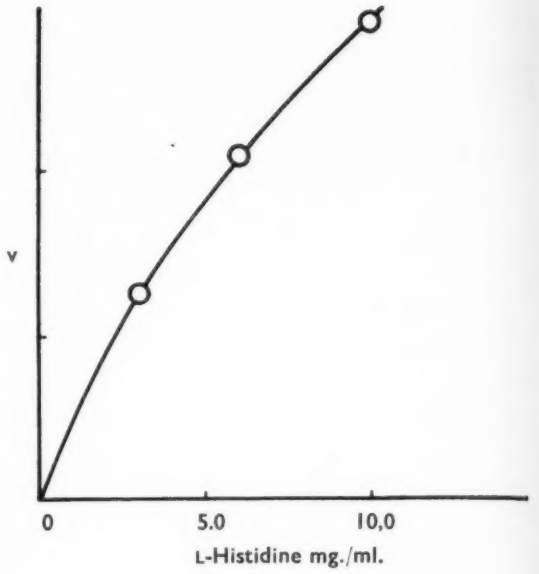


FIG. 1.—Influence of substrate concentration on histamine production by L-histidine decarboxylase in a guinea-pig kidney extract. Ordinate: v=initial rate of production of histamine (in arbitrary units). Abscissa: concentration of L-histidine in mg./ml.

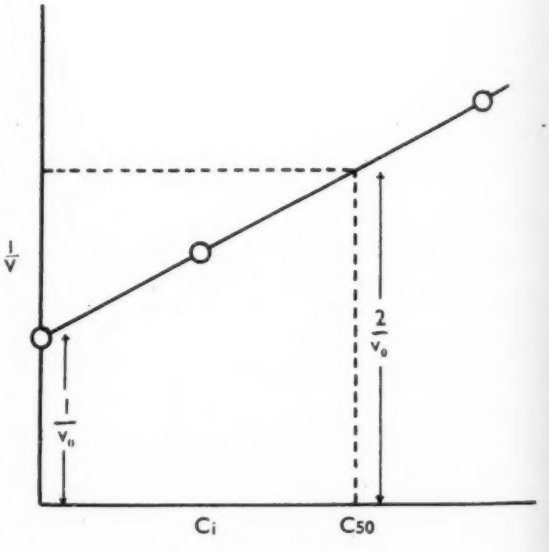


FIG. 2.—Inhibition of L-histidine decarboxylase in guinea-pig kidney extracts. Method of determining C_{50}. Ordinate: $\frac{1}{v}$ where v=initial rate of production of histamine (in arbitrary units). Abscissa: C_i=concentration of inhibitor in mg./ml. v_0=initial rate of the uninhibited reaction.

TABLE II

CONCENTRATIONS OF VARIOUS COMPOUNDS REQUIRED TO PRODUCE 50% INHIBITION (C_{50}) OF HISTIDINE DECARBOXYLASE

The concentration of L-histidine was 322×10^{-4} M, buffer *p*H 8·0, temperature 36°.

No.	Compound	$C_{50} \times 10^{-4}$ M
1	DL-α-Methyldopa	0·007
2	L-Dopa	0·18
3	L-2,4-Di-iodohistidine	40
4	D-2,4-Di-iodohistidine	40
5	L-Monoiodohistidine	50
6	DL-2-Methylhistidine	60
7	D-Histidine	86
8	DL-4-Methylhistidine	380
9	DL-β-(1-Methylimidazol-4-yl)alanine	420
10	L-β-(1-Methylimidazol-5-yl)alanine	910
11	Catechol	1·8
12	Salicylic acid	36
13	Phthalic acid	54
14	Imidazole	800
15	4-Methylimidazole	440
16	4-Methyl-5-nitroimidazole	34
17	Barbitone	330
18	L-Arginine	480
19	Creatine	2,000
20	Glycocyamine	2,600
21	Citrulline	3,200

DISCUSSION

The results given in Table II are grouped according to the chemical structures of the inhibitors. Compounds 18 to 21, though open-chain substances, were tested because they have some structural relationship to histidine. Although arginine was a fairly effective inhibitor, the other compounds were weak.

Substances 11 to 17 are all simple ring compounds with at least two electronegative atoms, either oxygen or nitrogen. Catechol was shown by Werle and Koch (1949) to be a potent inhibitor of histidine decarboxylase; this was confirmed. Of the imidazole analogues the 4-methyl-5-nitro-derivative was outstanding. Since the effect of the nitro-group is to withdraw electrons from the imidazole ring, thereby increasing the acidity of the nitrogen atoms in the ring, it is probable that this compound acts in a similar way to catechol. The acidic nitrogen atoms may be analogous to the acidic phenolic groups of catechol.

This idea may be tested quantitatively by calculating the concentrations of the anionic species present when the enzyme is 50% inhibited. Using the accepted *p*Ka values for catechol of 8.5 and 10.2, the concentrations of the mono- and di-valent catechol anions, present at *p*H 8.0 in a solution of catechol of concentration C_{50}, are approximately 45×10^{-6} M and 0.7×10^{-6} M respectively. For 4-methyl-5-nitroimidazole (*p*Ka = 9.9) the anion concentration at C_{50} is about 25×10^{-6} M. The charge of the 4-methyl-5-nitroimidazole anion can be partly distributed to the second nitrogen atom of the ring to form a species, obviously in lower concentration, analogous to the divalent catechol anion. However, since it was shown by Werle and Koch (1949) that phenol is a much less potent inhibitor than catechol, it is obvious that a simple mono-anion hypothesis is not acceptable, and that some part of the binding is due to the second electronegative atom.

Even with a pure compound several molecular species, dissociated or otherwise, are present in solution. Each species may contribute to the observed enzyme inhibition, and it cannot be assumed that they are all bound by the same mechanism, since both electrostatic interaction and hydrogen bonding are quite feasible. This may explain the behaviour of 4-methylimidazole in which the nitrogen atoms in the ring are more basic than in imidazole itself, and which, assuming electrostatic interaction, would be a weaker inhibitor than imidazole. 4-Methylimidazole was in fact somewhat more powerful than imidazole, and this could be due to the increased electron density in the nitrogen atoms resulting in greater ability of the undissociated base to form hydrogen bonds with the enzyme. As would be expected on the basis of either of the above hypotheses, barbitone was also an inhibitor of the enzyme.

Salicylic and phthalic acids, which may be regarded as derived from catechol by stepwise replacement of the phenolic groups by carboxyl groups, were progressively weaker inhibitors. This progressive decrease in potency may be due to steric factors, since the separation of the negatively-charged oxygen atoms of the ionized compounds increases in passing from catechol to phthalic acid. On the other hand, if hydrogen bonding is involved in the attachment of the ring to the enzyme, then interaction between the –C=O and –OH groups of the carboxyl-substituted compounds might lead to less efficient binding.

In the third group of inhibitors (Nos. 3 to 10, Table II) the iodohistidines were the most active, the acidity of the imidazole ring in these compounds being greater than in histidine itself. DL-2-Methylhistidine was surprisingly potent,

especially when compared with DL-4-methylhistidine, although in both compounds the methyl group would be expected to increase the basicity of the imidazole nitrogen atoms. D-Histidine, previously studied by Werle (1942), was also remarkably active. The D- and L-2,4-di-iodohistidines were prepared, and their potencies were found to be equal within experimental error. Further studies are required to elucidate the extent to which the D and L components contribute to the observed inhibitions by DL compounds. For this reason the relative activities of the two *N*-methylated histidines (Table II, Nos. 9 and 10) which were available only in the DL and L forms, respectively, were not strictly comparable. As expected, however, these were weak inhibitors, this result being analogous to the finding of Werle and Koch (1949) that phenol ethers are very weak inhibitors compared with the free phenols.

Werle (1942) found L-dopa to be a potent inhibitor of histidine decarboxylase. α-Methyldopa [DL-β-(3,4-dihydroxyphenyl)-α-methylalanine] was shown by Sourkes (1954) to be the most potent of a series of dopa analogues tested as inhibitors of dopa decarboxylase, and it also inhibits 5-hydroxytryptophan decarboxylase (Smith, 1960) and histidine decarboxylase (Udenfriend *et al.*, 1960). Quantitatively this compound (No. 1, Table II) was a considerably more potent inhibitor of histidine decarboxylase than is L-dopa itself (No. 2, Table II). This is not surprising since the α-methyl group increases the basicity of the α-amino-nitrogen atom, thus enhancing the ability of the latter to form a Schiff base with an aldehydic group, for example, that of pyridoxal 5′-phosphate.

It is likely that the views of Werle and Koch (1949) and of Metzler, Ikawa, and Snell (1954) regarding the mechanism of decarboxylation are basically correct. Histidine is probably attached to the holo-enzyme by the imidazole ring, the α-amino group, and the α-carboxyl group, the three-point attachment accounting for the optical specificity of the enzyme. However, since this specificity applies to the decarboxylation but not necessarily to the action of inhibitors, the actual interaction of carboxyl group and enzyme may not take place during sorption of substrate by the enzyme, but rather in an intermediate step preceding the subsequent breakdown to products. Although careful consideration of the results given here shows several discrepancies, it appears that increasing the acidity of the imidazole ring increases the binding of this portion of the molecule to the enzyme active centre.

The structural requirements of inhibitors of histidine decarboxylase and of dopa decarboxylase are clearly very similar. This similarity could mean that the enzymes are identical as implied by Udenfriend *et al.* (1960), but it might also arise from interaction of the inhibitors with the coenzyme, pyridoxal 5′-phosphate, which appears to be common to all decarboxylases.

2-Thiohistidine, 2-thioimidazole, ergothionine and cysteine were also tested as histidine decarboxylase inhibitors. The results are not included in Table II, because these compounds appear to act by a peculiar mechanism. Histamine production in the presence of these inhibitors was at first rapid, but decreased until, after about 2 hr., the enzyme was completely inactivated. Further studies on this type of compound are in progress.

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SOME PHARMACOLOGICAL ACTIONS OF SYNTHETIC ANALOGUES OF ANGIOTENSINAMIDE

BY

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In order to evaluate the importance of some structural features of asparagyl[1]-valyl[5]-angiotensin II (angiotensinamide) for its pharmacological actions, the relative potencies of angiotensinamide and five peptide analogues were studied on the blood pressure of the rat, the isolated rat uterus and the isolated guinea-pig ileum. All the modifications of the angiotensinamide structure that were studied led to a decrease of potency which, however, was not the same on all three preparations. The importance of the guanido group, the phenolic group and the length of the peptide chain for the pharmacological activities of these peptides is discussed.

The name *angiotensin* has been proposed by Braun-Menéndez and Page (1958) to replace the two trivial names, hypertensin and angiotonin, previously given to the product of renin action on the plasma α_2-globulins.

The knowledge of the structure of angiotensin peptides (Skeggs, Lentz, Kahn, Shumway and Woods, 1956; Elliott and Peart, 1957) and the development of methods for their synthesis (Rittel, Iselin, Kappeler, Riniker, and Schwyzer, 1957), as well as that of structural analogues, permitted the study of the relation of peptide structure to pharmacological activity. In a previous paper (Paiva and Paiva, 1960a) we have investigated the effect of modifications of the *N*-terminal grouping of valyl[5]-angiotensin II on its pressor and oxytocic activities, and concluded that a free β-carboxyl group in the *N*-terminal aspartyl residue, though not essential, is important for these two properties. A further study of the relation of angiotensin structure to activity was made possible by the availability of synthetic analogues prepared in the Ciba Laboratories. In the present paper we report a study of 6 such analogues, having in common an asparagyl instead of the aspartyl residue at the *N*-terminal end. Table I shows the amino acid sequences of valyl[5]-angiotensin II and of the 6 synthetic peptides studied. In order to simplify the reference to the different analogues, in this paper asparagyl[1]-valyl[5]-angiotensin II will be

TABLE I

THE SEQUENCE OF AMINO ACIDS IN VALYL[5]-ANGIOTENSIN II AND IN SIX SYNTHETIC ANALOGUES

The nomenclature used was derived from the name angiotensinamide (asparagyl[1], valyl[5]-angiotensin II) according to the rules proposed by Konzett and Berde (1959).

Peptide	Amino Acid Sequence
	1 2 3 4 5 6 7 8 9
Valyl[5]-angiotensin II	Asp.Arg.Val.Tyr.Val.His.Pro.Phe
Angiotensinamide	Asp(NH_2).Arg.Val.Tyr.Val.His.Pro.Phe
Isoleucyl[5]-angiotensinamide	Asp(NH_2).Arg.Val.Tyr.iLeu.His.Pro.Phe
Nitroarginyl[2]-angiotensinamide	Asp(NH_2).Arg.(NO_2).Val.Tyr.Val.His.Pro.Phe
Ornithyl[2]-angiotensinamide	Asp(NH_2).Orn.Val.Tyr.Val.His.Pro.Phe
Phenylalanyl[4]-angiotensinamide	Asp(NH_2).Arg.Val.Phe.Val.His.Pro.Phe
Homotyrosyl[4:5]-angiotensinamide	Asp(NH_2).Arg.Val.Tyr.Tyr.Val.His.Pro.Phe

referred to as *angiotensinamide*, and the name of the other peptides will be derived from this in the manner proposed by Konzett and Berde (1959) for the nomenclature of oxytocin analogues.

The pharmacological preparations used in the present study were the blood pressure of the rat, the isolated rat uterus and the isolated guinea-pig ileum. The first preparation was chosen because the pressor action is the most characteristic pharmacological action of angiotensin *in vivo*, and the two other preparations were chosen for being the most sensitive to angiotensin yet described (Ludueña, 1940; Picarelli, Kupper, Prado, Prado, and Valle, 1954; Schwartz, Masson, and Page, 1955).

Methods

The angiotensin analogues were all synthetic products prepared at Ciba Laboratories, Basle, and purified as free peptides in the final stage of the synthesis by counter-current distribution. The purity of the products was checked by paper chromatography and they were found to be either chromatographically pure or to contain small amounts (not more than 1%) of the corresponding aspartyl-peptides (formed by hydrolysis of the *N*-terminal asparagine amide group). All preparations contained 1 to 3 molecules of water and 1 to 2 molecules of acetic acid per molecule of peptide (Schwyzer, 1960).

Pharmacological Assays

Rat Blood Pressure.—Albino rats, weighing between 200 and 250 g., were anaesthetized with urethane 150 mg./100 g. body weight, and were kept on artificial respiration. The blood pressure was recorded with a mercury manometer connected to the carotid artery. Injections were made through a plastic cannula in a femoral vein. The rats were heparinized, and received an intravenous injection of hexamethonium bromide 5 mg./100 g. body weight. For the assay of *pressor activity* 0.1 or 0.2 ml. of the peptides, in isotonic solution, was injected.

Rat Uterus.—Virgin rats weighing 150 to 200 g. received subcutaneous injections of 10 μg. of stilboestrol per 100 g. body weight and were killed 16 to 20 hr. later by a blow on the head. The uterine horns were suspended in aerated de Jalon solution in a 2 ml. bath at 30°. The contractions were recorded with an isotonic lever with a six-fold magnification and a load of 1.0 to 1.5 g. In this paper, the activity of the peptides on this preparation is called *oxytocic activity*.

Guinea-pig Ileum.—Terminal segments of the ileum of guinea-pigs weighing 150 to 200 g. were suspended in a 2 ml. bath of aerated Tyrode solution at 35°. The contractions were recorded as described above for the rat uterus. The effect on the isolated guinea-pig ileum is called *gut-stimulating activity*.

The pressor, oxytocic and gut-stimulating activities of the 5 analogues of angiotensinamide were estimated by assays against standard solutions of angiotensinamide. For each analogue, a rough preliminary estimate of activity was made, and then approximately equipotent solutions of the peptide and of angiotensinamide were compared using a four-point design. The evaluation of potency was made by measuring the height of the recorded increases in blood pressure or contractions of the isolated smooth muscles, and submitting the data to the statistical treatment described by Schild (1942) for obtaining a potency ratio and fiducial limits. The relative potency of each peptide was defined as the ratio of the activity of one mole of peptide to the activity of one mole of angiotensinamide, which was arbitrarily taken as 100.

Results

All the peptides had detectable activity on the three pharmacological preparations. Qualitatively, no difference was detected between the pressor response to angiotensinamide on the rat and that of the other analogues. Although the oxytocic and gut-stimulating activities of isoleucyl^{5}- and homotyrosyl$^{4:5}$-angiotensinamides were qualitatively indistinguishable from angiotensinamide, the other 3 analogues produced a different type of response. On the isolated rat uterus, addition of nitroarginyl^{2}-, ornithyl^{2}- or phenylalanyl^{4}-angiotensinamide to the bath caused only a single contraction, instead of the rhythmic response characteristic of angiotensinamide (Paiva and Paiva, 1960b). On the isolated guinea-pig gut, the three last-mentioned analogues produced a tonus of shorter duration than that produced by

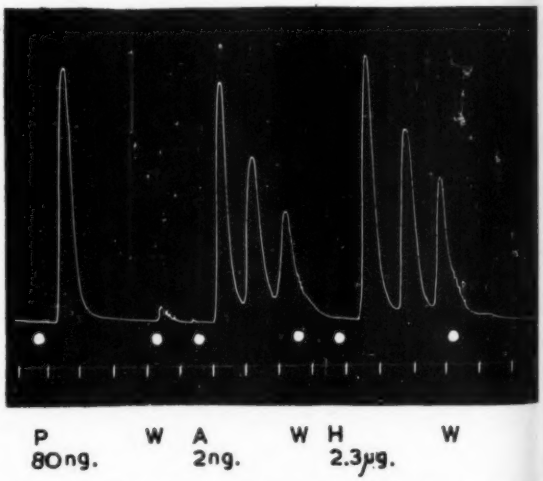

FIG. 1.—Isolated rat uterus suspended in a 2 ml. bath containing de Jalon solution at 30°. Responses to additions of phenylalanyl^{4}-angiotensinamide (P), angiotensinamide (A) and homotyrosyl$^{4:5}$-angiotensinamide (H). At W the preparation was washed three times. Time, min.

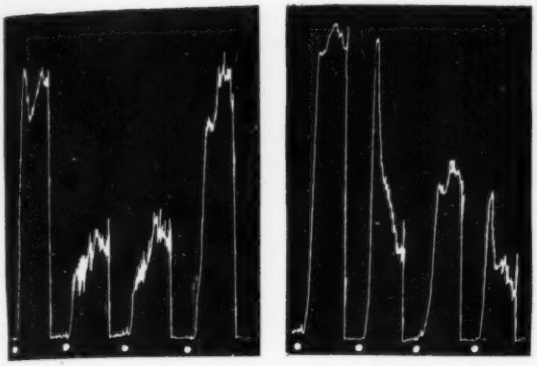

FIG. 2.—Isolated guinea-pig ileum suspended in a 2 ml. bath containing Tyrode solution at 35°. Contractions produced by angiotensinamide (A), homotyrosyl[4:5]-angiotensinamide (H), and phenylalanyl[4]-angiotensinamide (P).

angiotensinamide. To illustrate these qualitative differences, Figs. 1 and 2 show the response of the rat uterus and of the guinea-pig gut to angiotensinamide and to homotyrosyl[4:5]- and phenylalanyl[4]-angiotensinamide.

In spite of these qualitative differences, we decided to make a quantitative comparison of the oxytocic and gut-stimulating potencies of angiotensinamide with those of all the other peptides. The oxytocic activity was assessed from the height of the first contraction of the rat uterus, and the gut-stimulating activity from the maximum height of contraction of the ileum during the 90 sec. of contact with the drug. The results of the assays are summarized in Table II. No significant deviation of parallelism was found in any of the assays ($P>0.2$).

The possibility of competitive inhibition of angiotensinamide by homotyrosyl[4:5]-angiotensinamide was investigated in the three pharmacological preparations. The administration of mixtures of angiotensinamide with 100-fold concentrations of the homotyrosyl analogue resulted in simple summation of effects, without any sign of inhibition. Similar summation of effects was observed with mixtures of equipotent amounts of angiotensinamide with each of the other analogues; in these mixtures, the ratio between the molar concentration of the analogues and that of angiotensinamide ranged from 2, in the case of the pressor action of the mixture with isoleucyl[5]-angiotensinamide, to 350 in the case of the gut-stimulating action of the mixture with nitroarginyl[2]-angiotensinamide.

DISCUSSION

All the analogues studied were less active than angiotensinamide on the three pharmacological preparations. It is remarkable that relatively small alterations in the structure of angiotensinamide resulted in marked differences in the three pharmacological activities studied. One example of this is seen in Table II, where the ratios between the potencies of angiotensinamide and nitroarginyl[2]-angiotensinamide were found to be 4 for the pressor, 32 for the oxytocic, and 350 for the gut-stimulating activity. Another example illustrating that small changes in the structure of angiotensinamide resulted in marked alterations of pharmacological activity is where the presence of an extra methyl group, in isoleucyl[5]-angiotensinamide, resulted in a product that was about one-half to one-third as active as angiotensinamide. Furthermore, small activity was found for phenylalanyl[4]-angiotensinamide, which differs

TABLE II

RELATIVE POTENCIES OF SOME ANGIOTENSIN ANALOGUES ON THE RAT BLOOD PRESSURE, THE ISOLATED RAT UTERUS AND THE ISOLATED GUINEA-PIG ILEUM

Relative potencies were calculated on a molar basis, the value 100 being arbitrarily assigned to angiotensinamide. Figures in parentheses indicate the 95% fiducial limits.

Peptide	Rat Blood Pressure	Isolated Rat Uterus	Isolated Guinea-pig Ileum
Angiotensinamide	100	100	100
Isoleucyl[5]-angiotensinamide	52 (±8)	33 (±3)	35 (±5)
Nitroarginyl[2]-angiotensinamide	25 (±3)	3·1 (±0·2)	0·29 (±·05)
Ornithyl[2]-angiotensinamide	8·9 (±0·9)	1·6 (±0·2)	0·73 (±0·08)
Phenylalanyl[4]-angiotensinamide	4·9 (±0·8)	2·5 (±0·4)	3·2 (±0·2)
Homotyrosyl[4:5]-angiotensinamide	0·33 (±0·05)	0·10 (±0·02)	0·10 (±0·02)

from angiotensinamide only by the absence of one phenolic hydroxyl group. These differences, however, are not so surprising, in view of the reported discrepancies (Berde, Doepfner, and Konzett, 1957; van Dyke, 1959) in the potencies of oxytocin and vasopressin analogues towards different biological tests.

Some conclusions regarding the importance of the guanido group of angiotensinamide for its pharmacological activities may be drawn from the results obtained with nitroarginyl[2]- and ornithyl[2]-angiotensinamide. The nitroarginyl analogue, in which the ionization of the guanido group is blocked, had a pressor activity that was one-quarter that of angiotensinamide, but still almost three times that of the ornithyl analogue, in which the guanido group was replaced by an amino group. This suggests that the importance of the guanido group for the pressor activity of angiotensinamide may not be attributed entirely to its basic character. The same is not so evident with regard to the oxytocic activity, since the two analogues have very low oxytocic potencies, nitroarginyl[2]-angiotensinamide being still twice as active as ornithyl[2]-angiotensinamide. The gut-stimulating activity, on the other hand, seems to be more dependent on the basicity of the guanido group, since this activity is negligible in nitroarginyl[2]-angiotensinamide, whereas the ornithyl analogue is 2.5 times as potent. The inability of ornithine to replace arginine, especially in the case of the gut-stimulating activity, may be explained by the shortening of the side-chain interfering with the interaction of the basic group with the receptor. This explanation seems better than the one offered by Katsoyannis and du Vigneaud (1958) for the similar case of arginine and lysine vasopressins, namely, that the differences in the pK'_3 of the two amino acid residues might be responsible for the difference in the pharmacological activity of the two analogues. Since the two pK'_3 values are well above the physiological *p*H, the arginine guanido group and the ornithine amino group should both be completely ionized at that *p*H and, therefore, equally charged.

Homotyrosyl[4:5]-angiotensinamide, although inducing the same type of response as angiotensinamide, was the least active of the analogues. The absence of competitive inhibition of angiotensinamide by this analogue suggests that its low activity may be due to a disproportionate distance, introduced by the extra tyrosyl residue, between two points of attachment to the receptor.

After this paper had been submitted for publication we had knowledge of a paper by Gross and Turrian (1960), in which the pressor activity of the first four analogues of Table II were studied on the nephrectomized rat.

These authors found that the isoleucyl analogue was as active as angiotensinamide, and that the arginyl and ornithyl analogues were, respectively, one-half and one-quarter as active as angiotensinamide. However, no measure of the error involved in their assays is given, and it is impossible to determine the significance of the difference between our results.

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EVALUATION OF ANTAGONISTS OF HISTAMINE, 5-HYDROXYTRYPTAMINE AND ACETYLCHOLINE IN THE GUINEA-PIG

BY

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Changes in the resistance to entry of air into the lungs of anaesthetized guinea-pigs have been used to study the effects of histamine, 5-hydroxytryptamine, and acetylcholine. Some known antagonists of these bronchoconstrictor agents have been investigated. The degree of antagonism has been expressed in terms of the dose ratio, previously used only with *in vitro* preparations.

In 1940 Konzett and Rössler described a simple method of recording the degree to which the lungs of anaesthetized animals resist inflation. Since then the method has been used on many occasions to study physiological changes in resistance, the effects of drugs upon resistance and the action of drugs upon responses produced by agents known to change resistance.

Many workers have assumed that any change in resistance was produced by changes in bronchiolar tone, and this point has been discussed by Konzett (1956). In the present paper a more detailed study is made of the increased resistance to inflation produced in anaesthetized guinea-pigs by histamine, 5-hydroxytryptamine and acetylcholine and of the effects upon these responses of some of the antagonists of these drugs. A method for estimating the potency of these antagonists using the dose ratio (Gaddum, Hameed, Hathway and Stephens, 1955) has been employed.

METHODS

Although the method used was originally evolved for this study it has since been described in detail by Collier, Holgate, Schachter, and Shorley (1960). Resistance to the entry of air into the lungs of guinea-pigs anaesthetized with urethane (1.25 g./kg.) injected intraperitoneally was recorded by the Konzett and Rössler (1940) apparatus connected to the tracheal cannula, the air being supplied by a miniature Starling pump. After the resistance to inflation had been increased by a drug, it was returned to the original level by clipping for 10 sec. the tube leading to the recording apparatus, thus forcing the full stroke volume of the pump into the lungs. This reopened collapsed lung segments. Constancy of the base line resistance was obtained by small alterations in pump stroke volume. Anaesthesia was maintained by further intraperitoneal doses of 0.1 g. urethane. Drugs dissolved in normal saline were given either into the jugular vein in volumes of 0.05 to 0.4 ml. washed in with 0.5 ml. saline containing 10 i.u. heparin/ml. or by stomach tube in volumes up to 10 ml. washed in with 2 ml. saline.

Histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate and acetylcholine chloride were used as agonists. As antagonists, chlorpheniramine maleate, diphenhydramine hydrochloride, mepyramine maleate, 2-bromolysergic acid diethylamide bitartrate, lysergic acid diethylamide tartrate and atropine sulphate were employed. All doses are given in terms of the active acid or base.

RESULTS

Agonists

As a preliminary to experiments using antagonists, control experiments were carried out in which only the agonists were given. With all 3 agonists the response increased over the first 3 to 6 doses, and these were excluded from subsequent analyses and from times given for the duration of experiments. In these control experiments 4 doses in geometric progression were chosen to cover the range of recordable response. These were given at intervals of 5 to 10 (usually 8) min. in random order over a period of $5\frac{1}{2}$ to $6\frac{1}{2}$ hr. with intervals of 30 to 60 min. interpolated between some of the sets of 4 doses.

The response measured in all experiments was the maximum excursion of the piston recorder of the Konzett and Rössler apparatus. This was taken to indicate the maximum resistance to air entry. Preliminary investigations had shown this

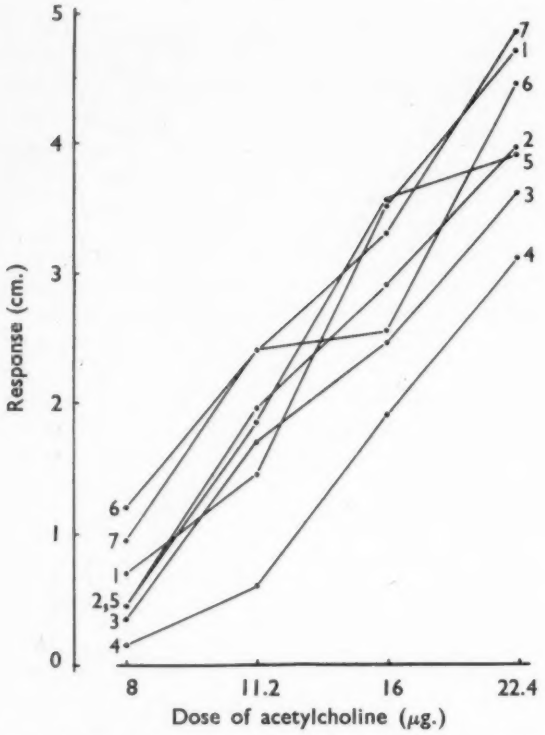

FIG. 1.—Log. dose-response curves for a control experiment with acetylcholine. Each line is drawn through points corresponding to 4 consecutive doses, and numbers indicate order of administration of each set of doses. Intervals of 30–60 min. were interpolated between sets 4, 5, 6, and 7. Responses were measured as the maximum excursion of the piston recorder in cm.

measure to be as informative as the area under the curve relating increase in excursion and time. The response was plotted against log dose of agonist as shown for an experiment on acetylcholine in Fig. 1.

Five such control experiments were carried out, 2 using histamine, 1 using 5-hydroxytryptamine and 2 using acetylcholine. From analysis of the results of these experiments it was found that, with 5-hydroxytryptamine and acetylcholine, the average dose-response curve was linear within the limits of experimental error. The deviations from linearity were significant in the experiments with histamine, but the biasing effect of this on the dose ratio (*vide infra*) was estimated as less than 4%.

In one experiment with histamine the slopes of the dose-response curves varied significantly as the experiment progressed, but in the remaining experiments with histamine and other agonists, differences in slope from one set of 4 responses to another were not significant.

In all control experiments the average level of response varied significantly between sets of responses. The degree of variation was expressed by determining the dose ratio for each set of 4 responses relative to the first set. Ideally this value should be unity since no antagonist had been given. The values obtained for the dose ratio are plotted against time in Fig. 2. In experiments involving acetylcholine the dose ratio showed progressive increase over a series of 4 sets of 4 doses with 8 min. intervals between doses and sets. In some preparations interpolation of

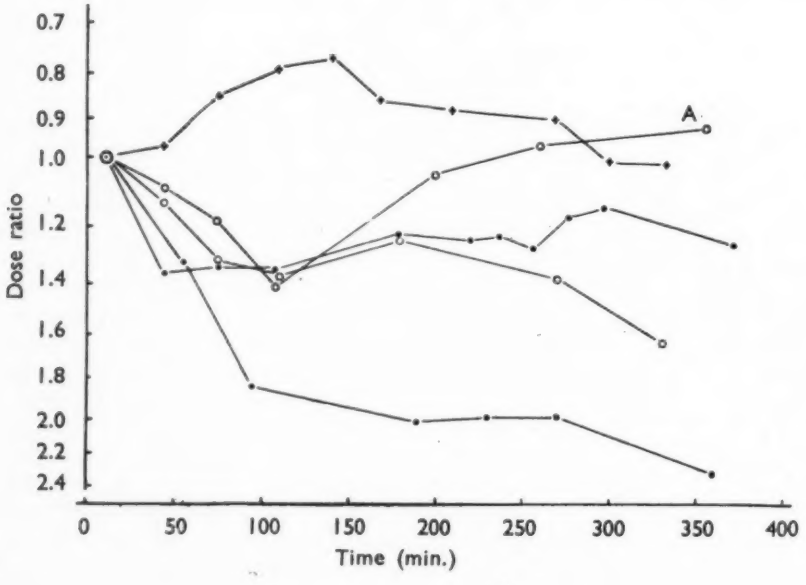

FIG. 2.—Control experiments. Dose ratios of sets of 4 responses relative to first set of 4 responses, plotted against time, taken as the mean for the period covered by a set of 4 observations. ●—● = Histamine; +—+ = 5-hydroxytryptamine; O—O = acetylcholine. Line A represents the results from the experiment illustrated in Fig. 1.

a 30 or 60 min. interval between 2 consecutive sets led to a decrease in dose ratio. Such a rest period did not, however, lead to a decrease in dose ratio in experiments with histamine.

Antagonists

Following Gaddum *et al.* (1955) the potency of an antagonist at a given concentration and a given time after administration is defined as the ratio A/A_0, where A_0 is the dose of the agonist producing a response (y) before the antagonist, and A is the dose of the agonist producing the same response (y) at the given time after the antagonist is administered. The dose ratio can be defined unambiguously only when the log. dose-response curves before and after giving the antagonist are parallel.

To obtain suitable data for the calculation of the dose ratio thus defined the following experimental procedure was adopted. Two doses of agonist were given repeatedly until the responses were stable. The antagonist was then given and the rise and fall of effect was followed by giving increasing and decreasing doses of agonist. Pairs of doses were chosen which, it was hoped, would produce responses comparable in magnitude to those obtained in the absence of antagonist. During the decline in the degree of antagonism intervals of up to 1 hr. were often allowed to elapse without doses of agonist being given. In the case of acetylcholine this was necessary in order to obtain maximal degree of recovery of the preparation (*vide supra*). When the dose ratio had fallen to 2 or less and stable responses were obtained to 2 doses of agonist, a further dose of antagonist was sometimes given.

From the data thus obtained the dose ratio could be determined graphically or by computation. The graphical method, suitable for routine investigation of antagonism, consists of plotting the responses against log. dose, estimating by eye the average slope within pairs of responses and drawing parallel lines through the centroid of each pair. The log. of the dose ratio is given by the horizontal distance between the parallel lines.

The computational approach, which is less subjective, has been used in all experiments reported here. The average slope within sets (usually a pair) over the duration of the experiment was calculated by standard regression procedures and the dose ratio determined from parallel lines with this slope fitted to sets of comparable responses.

In both methods where the degree of antagonism changes rapidly with time, individual responses may have to be used.

TABLE I

ANTAGONISM OF 5-HYDROXYTRYPTAMINE BY LYSERGIC ACID DIETHYLAMIDE

At 28 min. 50 μg./kg. lysergic acid diethylamide was given intravenously.

Dose No.	Time (min.)	Dose of 5-Hydroxy-tryptamine (μg.)	Response (cm.)
1	0	1	2·1
2	8	2	3·9
3	16	2	3·7
4	24	1	1·25
5	32	10	1·4
6	40	20	1·6
7	48	40	3·1
8	56	40	2·0
9	64	20	1·0
10	124	20	4·4
11	132	10	2·35
12	192	10	4·05
13	200	5	2·65
14	290	5	4·6
15	298	2·5	3·7
16	306	4	4·3
17	314	1	1·05
18	322	2	2·1

Table I gives the results of an experiment with 5-hydroxytryptamine and lysergic acid diethylamide; and Fig. 3 illustrates the method of determining from these data the dose ratio at different times.

Table II shows for 6 chosen antagonists dose ratios which are the maximal calculated during each experiment. When the antagonist was given intravenously the degree of antagonism often changed rapidly in comparison with the interval of 8 min. between doses of agonist. This may have resulted in underestimation of the maximal dose ratio.

Specificity of Antagonism

Specificity of antagonism was examined using diphenhydramine, mepyramine, lysergic acid diethylamide and atropine. Pairs of doses of each of the 3 agonists—histamine, 5-hydroxytryptamine and acetylcholine—were given at 5 min. intervals, usually in serial order with high and low doses alternating. When stable responses to each of these doses were obtained the antagonist was given intravenously. The sequence of doses of agonist was continued, increasing them where necessary to give responses

TABLE II

DOSE RATIOS CALCULATED FOR SIX ANTAGONISTS AT VARYING DOSES

Agonist	Antagonist	Dose (μg./kg.)	Route	Dose Ratio	No. of Experiments	Time at which Dose Ratio Measured (min)
Histamine	Chlorpheniramine	1·6	Intravenous	1·5	1	4
		4		1·6	1	16
		16		3·1	1	4
		32		2·8	1	8
		40		5·4	1	12
		77		16	1	32
		400		27	1	32
		400	Oral	3·2	1	24
		1,700		52–330	3	44–80
	Diphenhydramine	3–6	Intravenous	0·8–1·4	4	8–32
		33–40		1·3–1·6	3	8–16
		80		1·8	1	4
		165		3·7	1	16
		320–400		3·0–6·8	4	8–28
		800		6·6	2	4–12
		2,000		32	1	24
		1,700–1,800	Oral	1·3–2·7	2	32–48
		17,000–18,000		40–62	3	64–84
	Mepyramine	4	Intravenous	2·4–4·2	2	12–24
		40		12–28	3	24–40
		400		3,500	1	32
		1,750–1,800	Oral	2.0–9·0	4	56–80
		8,750–9,000		12–110	3	40–104
		17,500		730	1	88
5-Hydroxytryptamine	Bromolysergic acid diethylamide	1	Intravenous	0·9–1·0	3	8–16
		10		1·9–6·2	3	16–24
		100		10–23	3	24–40
		100	Oral	1·0	1	16
		400–500		1·9–2·8	2	56–102
		2,000		3·9–4·9	2	72–160
	Lysergic acid diethylamide	1	Intravenous	1·0–2·5	3	16–32
		10		4·8–7·9	3	24–32
		50		32–34	2	32
		100		25–55	3	32–40
		50	Oral	6·1–14	2	32–88
		100		15	1	100
Acetylcholine	Atropine	1	Intravenous	1·1–1·3	2	8
		10		3·7–4·2	2	8–12
		100		10–20	2	4–8
		1,000	Oral	3·0–6·3	2	20–50
		5,000		22	1	188
		10,000		49	1	186

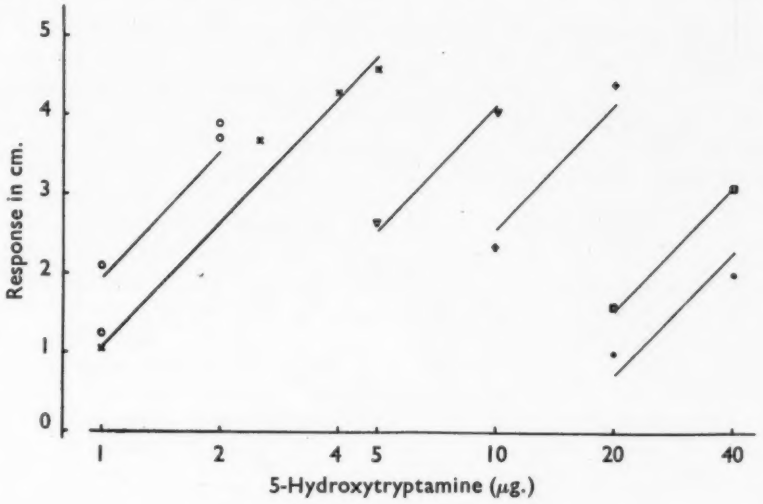

FIG. 3.—Log. dose-response curves illustrating data of Table I. O—O = Responses 1 to 4; □—□ = responses 6 and 7; ●—● = responses 8 and 9; +—+ = responses 10 and 11; ▽—▽ = responses 12 and 13; and X—X = responses 14 to 18. For the 5 sets of responses after lysergic acid diethylamide the dose ratios were 24, 34, 7.6, 3.8, and 1.5 at 16, 32, 100, 168, and 278 min. respectively after giving the antagonist.

TABLE III

DOSE RATIOS OBTAINED IN SPECIFICITY EXPERIMENTS

Dose ratios given are the maxima or minima observed.

Antagonist	Intravenous Dose (μg./kg.)	Histamine		5-Hydroxytryptamine		Acetylcholine	
		Dose Ratio	Time (min.)	Dose Ratio	Time (min.)	Dose Ratio	Time (min.)
Diphenhydramine	400	7·8	37·5	1·9	12·5	2·0	102·5
Mepyramine ..	40	15	12·5	0·98	25	1·6	37·5
Lysergic acid diethylamide ..	50	0·83	15	53	32·5	0·76	10
Atropine ..	100	2·8	22·5	1·4	12·5	18	27·5
,, ..	100	2·4	10	1·7	15	27	20

similar to those obtained in the absence of the antagonist. The dose ratios could then be calculated for each agonist independently. The results are shown in Table III, in which dose ratios of less than 1 indicate potentiation. The 2 experiments with atropine showed good agreement.

DISCUSSION

This work shows that methods of evaluating the potency of an antagonist previously used *in vitro* can be applied to an *in vivo* preparation provided that the response to the agonist remains sufficiently stable over an adequate period of time. We believe that this has been achieved by certain refinements of the technique first reported by Konzett and Rössler (1940). These include keeping the preparation at a fairly constant degree of anaesthesia and maintaining aeration near the original level by reinflation of collapsed lung segments and slight alterations in pump stroke volume.

Sufficient stability of response was indicated in the control experiments previously described and illustrated in Fig. 1. The greatest loss of response resulted in a dose ratio of 2.3 over a period of 6 hr. Over shorter periods of time corresponding to times of maximal antagonism in Table II the change of response was always considerably less. The evidence of these control experiments is supported by many experiments in which antagonists have been given and full recovery of response observed after 5 to 6 hr. when the effects of the antagonists have disappeared.

When an antagonist has been given, the slopes of the log. dose-response curves before and after its administration have usually been comparable, the exception being when high doses of lysergic acid diethylamide were used. In this case the slope was decreased and it was impossible to calculate dose ratios.

The results given in Table II provide a basis for studying the variation in dose ratio between

animals given the same dose of antagonist. This variation is best measured by the coefficient of variation which is estimated from these results as about 35% for drugs given intravenously and about 70% for those given by mouth. Presumably this larger figure reflects variation in absorption of the antagonist.

From these results in an *in vivo* preparation, we can study the relative potency of a drug given intravenously and by mouth. In this short series of drugs remarkable differences can be seen as shown in Table IV. Even among the 3 antihistamines and the 2 antagonists of 5-hydroxytryptamine great differences exist.

TABLE IV

RATIOS OF ORAL TO INTRAVENOUS DOSES GIVING EQUAL DEGREES OF MAXIMAL ANTAGONISM

Antagonist	Approximate Ratio
Chlorpheniramine	4
Diphenhydramine	6
Mepyramine	200
Bromolysergic acid diethylamide..	100
Lysergic acid diethylamide ..	4
Atropine	60

In the specificity experiments given in Table III, dose ratios obtained against the primary agonist are reasonably comparable with those given in Table II, indicating that the use of other agonists in these experiments does not modify the primary antagonism. Both antihistamines showed specificity although this was greater for mepyramine than for diphenhydramine. Both antagonized acetylcholine to some extent, but the maximum dose ratio was obtained at a later time than that for histamine. The results for lysergic acid diethylamide were interesting in that the compound appeared to potentiate histamine and acetylcholine. This finding confirmed a previous experiment in which the responses before and after lysergic acid diethylamide were not sufficiently comparable to enable the dose ratio to be calculated.

In the 2 experiments using atropine, both histamine and 5-hydroxytryptamine were antagonized to some extent though the dose ratio is rather greater for histamine. This contrasts with the finding of Cambridge and Holgate (1955), using guinea-pig ileum, and suggests that 5-hydroxytryptamine is probably not acting by the liberation of acetylcholine. One would speculate that the 2 types of receptor suggested by Gaddum and Picarelli (1957) are not present in the bronchioles.

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THE TRYPANOCIDAL ACTION OF HOMIDIUM, QUINAPYRAMINE AND SURAMIN

BY

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Homidium, quinapyramine, and suramin (Group II compounds) produce their trypanocidal effect *in vivo* only after a latent period of 24 hr. or more, during which time the trypanosomes may continue to multiply; this is in contrast to trivalent arsenical and diamidine compounds (Group I compounds), which begin to act immediately. Group II compounds also differ from Group I compounds in that (*a*) they have only a slight tendency to combine with trypanosomes, (*b*) they have little trypanocidal action *in vitro*, but (*c*) they make trypanosomes non-infective to fresh subinoculated mice. To explain these features it is postulated that homidium, quinapyramine, and suramin first combine in small amounts with some receptor on the trypanosome and then block some biochemical system which produces a hypothetical substance X which is needed for cell division of the trypanosome; the trypanosome is supposed to contain a preformed store of this substance X sufficient for several divisions to take place; and it is only when this store is exhausted that cell division is prevented and the trypanosome eventually dies.

The purpose of this paper is to describe certain characteristics of the trypanocidal action of homidium (Ethidium ; B.Vet.C.Supp. 1959, p. 27), quinapyramine (Antrycide ; B.Vet.C., 1955, p. 553), and suramin, and to offer a hypothesis to explain them. These compounds differ from other trypanocidal compounds such as trivalent arsenicals and diamidines in that their trypanocidal action *in vivo* is manifested only after a long latent period. Experiments were undertaken to study quantitatively the behaviour of the trypanosomes during this latent period.

METHODS

Rats or mice were infected with trypanosomes by intraperitoneal inoculation When the blood contained scanty trypanosomes, blood from the tail was diluted in a W.B.C. or R.B.C. pipette with a fluid which lysed the erythrocytes and stained the trypanosomes ; the trypanosomes were then counted in a haemocytometer slide under the microscope. A suitable fluid for this purpose was: 1% methylene blue, 2 ml. ; glacial acetic acid, 0.25 ml. ; water, 50 ml. In the case of *Trypanosoma evansi*, the number of trypanosomes in a drop of blood from the tail was probably the same as that in the main volume of circulating blood. *T. congolense*, on the other hand, tends to accumulate in the capillaries of the tail, and the count recorded may often have been much greater than that in the circulating blood ; presumably, however, the two were proportional, and this discrepancy would not affect the general argument. The animal was then treated intraperitoneally with a dose of the drug approximately double the dose which was sufficient to remove all visible trypanosomes from the blood within four days. Counts of the trypanosomes were made at suitable intervals (usually morning and evening) until no further trypanosomes were found.

The trypanosomes used were *T. evansi*, Mathura strain, described in a previous paper (Sen, Sharma, and Hawking, 1960) which was studied in rats ; and *T. congolense* (N.I.M.R. strain) which had been maintained in mice for some years and which was very sensitive to quinapyramine and homidium.

RESULTS

The results of typical experiments are shown in Fig. 1 in which the time is plotted horizontally and the logarithm of the number of trypanosomes in the blood is plotted vertically. In the case of *T. evansi*, the trypanosomes of the untreated control rat increase about 25 times (average) every 24 hr., that is, 4.7 divisions per 24 hr., or 1 division approximately every 5 hr. In the case of *T. congolense*, the trypanosomes of untreated control mice increased approximately 8 times every 24 hr, that is, approximately 1 division

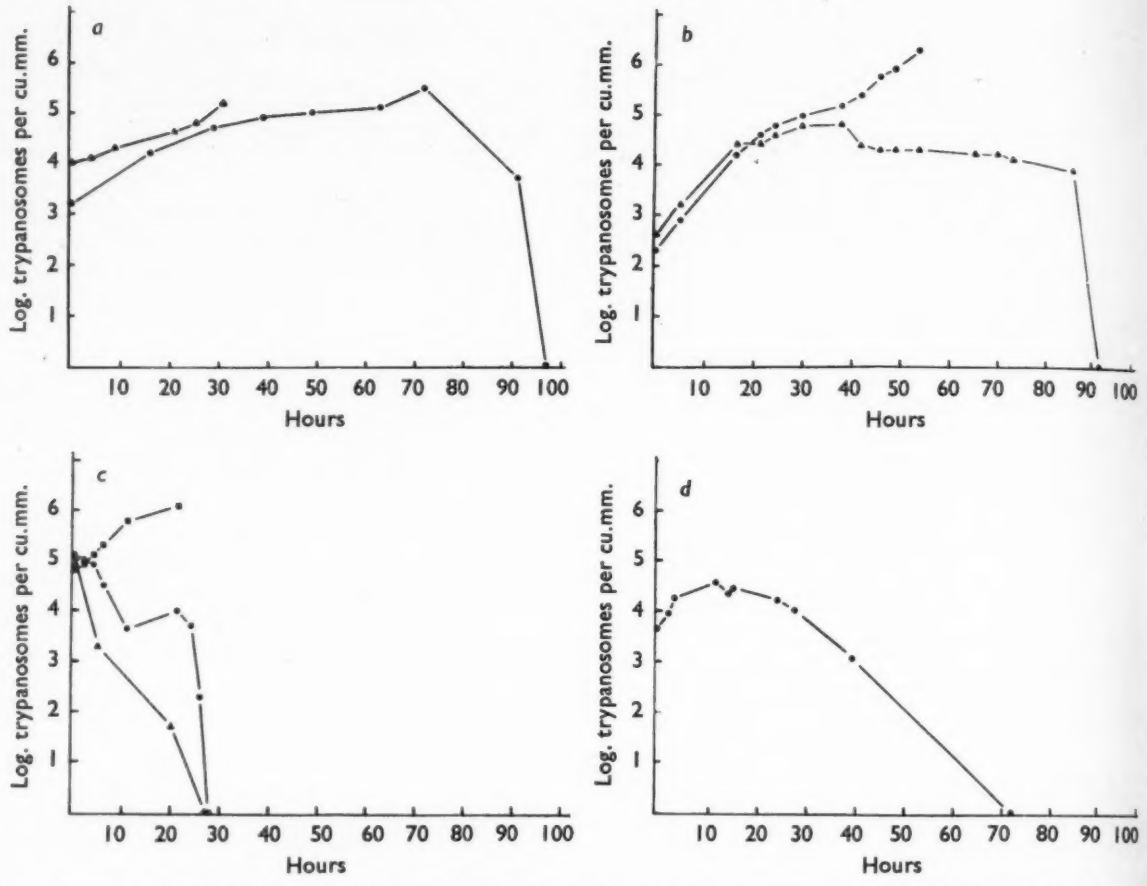

FIG. 1.—The vertical scales show the log. of the number of trypanosomes per cu.mm. tail blood; failure to see trypanosomes is arbitrarily indicated as log. 0. The horizontal scales show the time in hours after treatment. Drugs were given by intraperitoneal injection. Suramin 0.03 mg./100 g. ●—● (control ▲—▲) in (*a*). Quinapyramine 0.01 mg./100 g. ▲—▲ (control ●—●) in (*b*); melarsoprol 0.01 mg./100 g. ▲—▲, stilbamidine 0.2 mg./100 g. ●—● (control ■—■) in (*c*); were tested on *T. evansi* in rats. Homidium 0.004 mg./20 g., ●—●, in (*d*), was tested on *T. congolense* in mice.

every 8 hr. After treatment with two compounds (melarsoprol and stilbamidine), which are believed to exert a direct lethal action on the trypanosomes, the number of trypanosomes begins to fall immediately after the treatment, and reaches zero in about 27 hr. After treatment with homidium, quinapyramine, and suramin the trypanosomes continue to multiply for 24 to 72 hr.; then there is a period during which the trypanosomes remain approximately constant in number; finally they diminish rapidly and disappear. Depending on the dose, the drug, and the sensitivity of the trypanosome, these three phases vary in duration, but they can still be seen even when the dose is raised considerably. The essential point is that the trypanosomes continue to multiply for a considerable period after they have come into contact with the drug. In the case of suramin and quinapyramine the number may increase by 128 times, that is, 7 divisions; in the case of homidium it increased 8 times, that is, 3 divisions.

DISCUSSION

Two types of trypanocidal compounds can be recognized, according to their biological action as summarized in Table I.

It is considered that the first group—trivalent arsenicals (for example, trivalent tryparsamide, melarsoprol), acriflavine, diamidines (for example, stilbamidine, berenil)—are specifically absorbed by the trypanosomes, so that a high concentration is reached inside the cells, and that they then cause the death of the trypanosomes by direct action on some vital mechanism (Hawking,

TABLE I

TYPES OF TRYPANOCIDAL COMPOUNDS

	Group I Trivalent Arsenical Compounds	Group II Homidium and Other Phenanthridines, Quinapyramine, Suramin
Absorption by trypanosomes	Marked and immediate	Inconspicuous
Trypanocidal action *in vitro*	Marked	Slight or absent
Effect on infectivity ..	Slight	Marked
Trypanocidal action *in vivo*	Immediate	Manifest only after a period of multiplication

1938). The other compounds (homidium, quinapyramine, and suramin) are less simple in their mode of action.

Little absorption of the compound by the trypanosome, either *in vitro* or *in vivo*, can be shown by direct chemical methods, although a slight absorption probably does occur. Suramin could not be shown to be absorbed by trypanosomes, when a somewhat insensitive method was used (Hawking, 1939). Prothidium (a phenanthridine compound with pyrimidine side chain) is absorbed to a slight extent according to Taylor (1960). By contrast trivalent arsenicals and acriflavine are absorbed by trypanosomes in large amounts (Hawking, 1938) and the same is true of stilbamidine (Hawking, 1944; Fulton and Grant, 1955); this absorption can easily be demonstrated *in vitro* and *in vivo* and can be studied quantitatively.

The trypanocidal action *in vitro* of homidium and quinapyramine is moderate and that of suramin is slight. Suramin kills *T. rhodesiense* (old laboratory strain) (37°, 24 hr. exposure) only at 1/3,000 (Hawking, 1939). Under the same conditions quinapyramine kills *T. equiperdum* at 1 in 1 to 4 million (Hawking and Thurston, 1955) and dimidium kills *T. rhodesiense* (old laboratory strain) 1 in 1 million (Lock, 1950). These concentrations are all greater than are reached *in vivo* after therapeutic doses. By contrast the trivalent arsenicals and similar compounds under the same conditions kill trypanosomes at low concentrations like 1 in 50 million or 1 in 200 million.

When trypanosomes are exposed *in vivo* or *in vitro* for several hours to homidium, quinapyramine or suramin in suitable concentrations the trypanosomes appear unharmed and may continue to wriggle actively for 24 hr.; but if they are inoculated into fresh animals, no infection develops, showing that some profound change has been produced in them (Hawking, 1938; Lock, 1950; Ormerod, 1951; Hawking and Thurston, 1955). By contrast, when trypanosomes are exposed to arsenicals and similar compounds, their infectivity is not usually diminished, unless all the trypanosomes have been obviously killed or damaged before they are inoculated.

As demonstrated above, the *in vivo* trypanocidal action of homidium, quinapyramine, and suramin is exerted only after a latent period during which 3 to 7 cell divisions may occur, while the *in vivo* trypanocidal action of trivalent arsenicals and diamidine is manifested almost immediately.

It is suggested that these special features of the action of Group II compounds might be explained by a hypothesis on the following lines.

The drug first combines in small amounts with some receptor on the trypanosome. It then blocks some biochemical system which produces a hypothetical substance X which is needed for the cell division (multiplication) of the trypanosome. It is postulated that the trypanosome contains a preformed store of this substance X sufficient for several divisions to take place; when this store is exhausted, no further divisions can occur (perhaps the cell cannot even continue to function), and after a further period all the trypanosomes die.

The chief evidence for combination between group II drugs and trypanosomes (fixation) is that if trypanosomes are exposed to these drugs *in vitro* or *in vivo* they become non-infective to fresh animals, although they continue to wriggle for long periods. In its early stages part of this combination is probably reversible, but, judging by the period of exposure needed to make most of the trypanosomes non-infective, an irreversible combination seems to occur with dimidium in 0.5 hr. (Lock, 1950), with quinapyramine in 2.5 hr. (Ormerod, 1951), and with suramin in 1 to 2 hr. (Hawking, 1939). Newton (1957) found that *Strigomonas oncopelti* exposed to homidium (10 μg. per ml.) absorbed 3 μg. per 10^8 organisms in the first 3 hr., and that this combination was irreversible.

The phenomena of drug resistance show that homidium, quinapyramine, and suramin do not all act on the same site in the trypanosome (although there may be some overlapping between homidium and quinapyramine). Accordingly it may be supposed that either the compounds are

first fixed on different receptors (drug resistance depending on a diminished avidity of such receptors) after which all three act on the same biochemical system; or homidium blocks the production of a substance X, quinapyramine blocks the production of a similar substance Y, and suramin the production of a substance Z.

Substance X (or Y or Z) might be imagined to be a compound concerned with the synthesis of ribonucleic acid. In this connexion it may be noted that homidium rapidly inhibits the synthesis of deoxyribonucleic acid in *Strigomonas oncopelti* while the synthesis of ribonucleic acid and protein continues for several hours after addition of the drug (Newton, 1957). McIlwain (1946) has pointed out that in bacteria the molecules of many important substances, such as vitamins and co-enzymes, are limited in number per cell, for example, a cell may contain only 200 to 1,200 molecules of folic acid, and possibly only a few molecules of certain fundamental enzymes. Something the same may be true of trypanosomes, and substance X may be one of the molecules which are present only in limited numbers.

According to fluorescent appearances quinapyramine (Ormerod, 1951) and prothidium (Taylor, 1960) usually collect in the kinetoplast and in granules, in the cytoplasm of trypanosomes, and not in the nucleus to any large extent. (A similar distribution is shown by stilbamidine and acriflavine, which kill trypanosomes in a more direct manner). Either the hypothetical biochemical processes blocked by quinapyramine and prothidium take place mainly in the kinetoplast rather than in the nucleus, or only a small proportion of the drug absorbed by the trypanosome is responsible for the death of the organisms, the greater proportion being diverted into inactive combinations.

The morphological effects produced on trypanosomes by the administration of homidium, quinapyramine, or suramin to infected rats consist of: (i) Diminution in the percentage of dividing forms. (ii) Sometimes the occurrence of giant multinuclear trypanosomes in which the nucleus has divided once, or twice, but the cytoplasm has not; this is evidence of disordered cell division, but the disorder is greater in the cytoplasm than in the nucleus: this occurs with *T. rhodesiense* but it is rare with *T. equiperdum* or *T. congolense*. (iii) The appearance in the trypanosome cytoplasm of basophilic inclusion bodies which are best developed after 24 hr. Apparently these contain ribonucleic acid protein and drug (Ormerod, 1951). (iv) Diminished mobility in the trypanosomes, which can be seen under the microscope to wriggle less actively than usual (Mr. P. Walker, unpublished observation).

In discussing the mode of action of homidium upon *S. oncopelti* with particular reference to the metabolism of ribonucleic acid and deoxyribonucleic acid, Newton (1957) postulates that there is first a rapid combination of the drug with "primary binding sites" which does not interfere with growth and cell division, and then a slower combination with "secondary binding sites" which become available to the drug during growth; it is this secondary uptake which progressively inhibits growth. The conception of a rapid combination with primary binding sites (fixation), followed by a slower combination with secondary binding sites, is supported by many phenomena in trypanocidal action, and it is generally accepted. The suggestion that these secondary binding sites for homidium, etc., only become available during growth is an alternative hypothesis to the one submitted above in the attempt to explain the biochemical mechanism by which a drug can produce its inhibitory effect only after a latent period which may be 24 hr. or more. Whatever hypothesis is eventually proved to be correct, it is to be expected that it should apply to quinapyramine and suramin as well as to the phenanthridine compounds.

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THE EXCRETION OF SODIUM 6-(2,6-DIMETHOXYBENZAMIDOPENICILLANATE) MONOHYDRATE IN RATS

BY

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The excretion of sodium 6-(2 : 6 dimethoxybenzamido) penicillanate monohydrate has been studied. When injected intramuscularly it was rapidly excreted in the bile and urine of rats, but only in trace amounts in the faeces. The excretion products in the bile (2 to 3% of the dose) and urine (26–84% of the dose) are microbiologically active. The product in the bile may be in conjugated form.

Sodium 6-(2:6 dimethoxybenzamido) penicillanate monohydrate (BRL 1241) is a synthetic derivative of 6-aminopenicillanic acid which is almost immune to the action of penicillinase and has marked bactericidal activity against strains of *Staphylococcus aureus* resistant to natural forms of penicillin (Rolinson, Stevens, Batchelor, Cameron-Wood, and Chain, 1960).

Pharmacological studies in patients (Knudsen and Rolinson, 1960; Stewart, 1960) have shown that BRL 1241 is rapidly excreted in the urine in amounts up to about 75% of each injected dose. In laboratory animals (Brown, personal communication) about 30% is excreted in 6 hr. Of the remainder, a proportion can be detected in bile (Stewart, 1960), but there is no exact data on the amounts excreted by different routes.

The present study describes the mode and rate of excretion of BRL 1241 in bile, compared with urine and faeces.

METHODS

BRL 1241 was injected intramuscularly as a sterile neutral solution in water in doses of 100 and 300 mg./kg. Standard solutions for assay were made up from the pure amorphous powder.

Rats.—Male and female albino rats, 250 to 400 g. weight, bred in these laboratories, were used. The bile duct was cannulated at laparotomy, under ether and/or nembutal anaesthesia, with polythene tubing (0.50 mm. bore × 0.20 mm. wall) through which bile was collected into small graduated tubes for measured intervals of time. After recovery from the anaesthetic, the rats were restrained in close-fitting, flexible wire-mesh cages.

Assays.—BRL 1241 was assayed in test and standard solution on square agar plates 33 × 33 cm. against *Sarcina lutea* and the Oxford strain of *Staphylococcus aureus*. Standards were normally prepared in water, but, in some experiments, normal rat bile, plasma and urine were used as solvents. Concentrations of the drug in test fluids were estimated by interpolation on graphs relating inhibitory zone-diameter to the logarithms of concentrations of standards. Chromatographic bio-assay was performed by the ascending technique on 25 cm. square Whatman No. 1 paper. The solvent was butanol:ethanol:0.05 M phosphate 25:10:20, at *p*H 7.0. After a 17-hr. run, chromatograms were dried with a current of air at room temperature, cut into 7 mm. strips, and placed on seeded agar plates for overnight incubation. By this technique, BRL 1241 gave a mean R_F value of 0.91 ± 0.07.

Solutions for assay were kept at 4° or lower, and standards were made up freshly from a pure, stable preparation of BRL 1241 in powder form; when made up, these standard solutions were kept at −20°.

Urine and faeces from rats were collected over 24-hr. periods in metabolism cages. Urine was measured and assayed directly after dilution in water to 1:100 or higher. Faeces were weighed, homogenized in water with minimal dilution, and filtered. Before assay, urine and faeces were heated to 75 to 80° and kept at this temperature for 10 min. to minimize contamination. There was a fractional (15 to 20%) loss in the activity of BRL 1241, but this varied according to *p*H and other factors and no allowance has been made for it in the assay figures in the tables.

For the assays liver was washed clear of blood, blotted, weighed and homogenized in water. The homogenate was centrifuged and the supernatant inserted directly into cups in agar, without dilution, as with serum or plasma.

RESULTS

BRL 1241 was regularly present in the bile of healthy adult rats of both sexes in relatively high concentrations within 1 hr. of an intramuscular injection. After 100 mg./kg., 0.14 to 0.4 mg. was excreted in the first hour and further comparable

quantities in the second hour. Thereafter, the amount excreted dropped sharply and was hardly detectable after 4 hr. (Fig. 1). Excretion began within 10 min. of an injection, but the rate of excretion thereafter varied according to the dose injected. At 100 mg./kg. the peak values occurred between 30 and 90 min., whereas 300 mg./kg. gave sharper and disproportionately higher peak values at about 30 min. (Fig. 2). The actual concentrations of these peaks (0.60 mg./ml. and 2.6 mg./ml. respectively) were much higher than corresponding levels in the serum and in the liver (Table I).

At this rate of excretion in bile 0.32 to 1.08 mg. BRL 1241 was transferred to the duodenum during the 3 hr. after an injection of 100 mg./kg., equivalent to about 1 to 3.5% of the total dose or a mean excretion of 2.6 mg./kg. (Table II). After 300 mg./kg. the excretion in one rat was

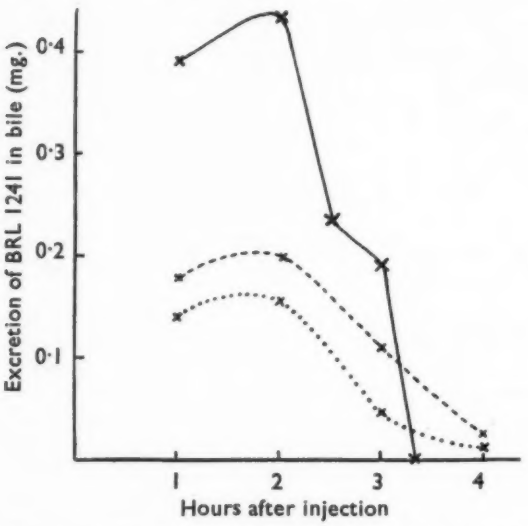

FIG. 1.—Rate of excretion of BRL 1241 in the bile of rats. —— Rat 1, 300 mg./kg. - - - - Rat 2, 100 mg./kg. . . . . Rat 3, 100 mg./kg.

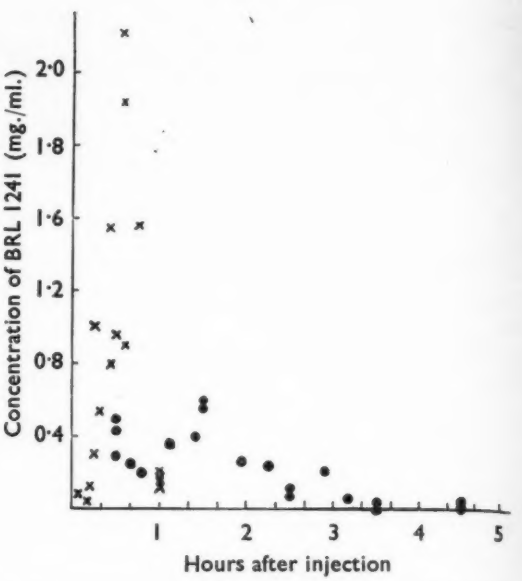

FIG. 2.—Concentration of BRL 1241 in the bile of rats injected intramuscularly. ✕ 300 mg./kg. ● 100 mg./kg.

TABLE I

CONCENTRATIONS OF BRL 1241 IN LIVER, SERUM, BILE AND URINE OF RATS INJECTED WITH 100 MG./KG.

Hours after Dose	BRL 1241			
	Serum mg./ml.	Liver mg./g.	Bile mg./ml.	Urine mg./ml.
1·5	0·029	0·013	0·420	9·320
3·5	0·005	0·002	0·110	10·300

TABLE II

EXCRETION OF BRL 1241 IN BILE, URINE, AND FAECES OF RATS

Dose (mg./kg.)	No. of Rats	24-hour Excretion of BRL 1241				Total (% of Dose)
			Bile	Urine	Faeces	
300	3	Mean (mg./kg.)	10·6	120	Trace	40–46
		% of dose	3·6	40		
100	14	Mean (mg./kg.)	2·6±0·98	53±23	0–0·2	27–86
		% of dose	1·1–2·7	26–84	0–0·2	

5.1 mg., nearly 6% of the dose, the mean excretion being 10.6 mg./kg.

Chromatographic bio-assays show that BRL 1241 had a lower R_F in bile than in water, irrespective of whether it was excreted in the bile *in vivo* or added *in vitro* (Table III). When incorporated *in vivo*, the drug appeared to be relatively more active against *Staph. aureus* than against *Sarcina lutea* (Table IV).

TABLE III
CHROMATOGRAPHIC BIO-ASSAY OF BRL 1241. R_F VALUES IN WATER AND BILE

BRL 1241	No. of Tests	R_F Values		
		Range	Mean	S.D.
Aqueous	8	0·89–0·93	0·91	0·07
Excreted in bile ..	10	0·66–0·85	0·78	0·08
Added to bile ..	8	0·66–0·88		

TABLE IV
ASSAYS OF BRL 1241 IN BILE AGAINST *SARCINA LUTEA* AND THE OXFORD *STAPHYLOCOCCUS*

Sample of BRL 1241	Expt. No.	Concentration (μg./ml.) as Assayed Against	
		Sarcina lutea	Oxford *Staph.*
Excreted in bile	1	450	2,000
	2	530	1,600
	3	210/290	1,550/1,000
	4	260/270	800/780
	5	260/375	975/990
	6	320/255	360/400
Added to bile ..	1	18·5	22·5
	2	18·0	18·5
Added to bile and incubated	1	25·5	28·5
	2	20·0	20·0
	3	10·0	10·0

In urine, the mean excretion after a dose of 100 mg./kg. was 53 mg./kg., the range being 26 to 84% of the injected dose (Table II). Samples obtained during the 4 hr. after injection gave assay values of 25 mg./kg. or more, indicating that excretion occurred rapidly, as in bile. Individual rats showed considerable variation in the quantities of drug detectable in urine. This appeared to be due to differences in urinary flow and to partial inactivation of BRL 1241 in acid solution, especially in 24 hr. samples. A further 15 to 20% loss occurred in heating urine (and faeces) at 75°, which was necessary to minimize contamination of the assay-plates. If allowance was made for these variables, it was clear that the bulk of the injected dose was rapidly excreted in the urine.

In the faeces, BRL 1241 was absent or barely detectable (Table II), accounting at maximum for only 0.2% of the injected dose.

DISCUSSION

It is known that BRL 1241 is rapidly excreted in the urine, in man as in laboratory animals (Knudsen and Rolinson, 1960; Douthwaite and Trafford, 1960; Stewart, 1960; Brown, personal communication). The present results show that excretion also occurs in bile, in rats, with comparable rapidity. The amount detectable in bile (usually 2 to 3% of the injected dose) is small compared with that in the urine, but is sufficient to produce concentrations (0.32 to 5.1 mg./ml.) which are in excess of those (0.001 to 0.005 mg./ml.) at which the drug is bactericidal to pyogenic cocci. Quantities of this order are above the levels present in serum and in the liver, so that the drug is concentrated 10 to 100 fold in the biliary tract and is presumably excreted directly after passage through the hepatic cells, without storage. The moiety of drug thus transferred to the intestine is presumably re-absorbed or inactivated, as little or none is detectable in the faeces.

Chromatographic studies have shown (Stewart, 1960) that BRL 1241 is excreted in human urine essentially unchanged. This is also true when the drug is excreted by rats in the urine but not in bile. The biliary excretion product shows changed chromatographic and microbiological properties. The rapid rate of excretion, and the absence of a metabolite in the urine, make it unlikely that the change is due to the formation of a hepatic metabolite, though it may be a physical conjugation with a substance present in the bile. Whatever the explanation, the resulting compound apparently retains full antibacterial activity, and may even show an apparent enhancement of activity against *Staph. aureus*.

BRL 1241 has also been found, in active form and in high concentration, in human bile (Stewart, 1960). In this respect it is similar to natural forms of benzylpenicillin.

BRL 1241 was kindly supplied by Beecham Research Laboratories.

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THE MECHANISM OF THE INHIBITORY EFFECT OF GLUTEN FRACTIONS ON THE PERISTALTIC REFLEX

BY

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The mechanism of the inhibitory effect of gluten fractions on the peristaltic reflex was studied using, first, a filtrate of the autoclaved peptic-tryptic digest of gluten (fraction III A.F.) and, second, the ultrafiltrate of an aqueous extract of gluten (fraction G.U.F.). The fractions did not act as antagonists to naturally-occurring agents such as acetylcholine or substance P, but they did depress the twitch response of the isolated guinea-pig jejunum preparation stimulated coaxially. The output of acetylcholine from cholinergic nerve endings in gut was decreased during rest as well as during electrical stimulation. In large amounts, the gluten fractions depressed acetylcholine synthesis *in vitro*. It was concluded that the mechanism of inhibition of the peristaltic reflex by gluten fractions was chiefly by decreasing acetylcholine release.

Intolerance to dietary wheat gluten has been established as a major factor in the pathogenesis of coeliac disease as well as of idiopathic steatorrhoea. Certain fractions of gluten depress the peristaltic reflex of isolated loops of rat jejunum (Schneider, Bishop and Shaw, 1960). The present work is an attempt to analyse the mechanism of this depression of the peristaltic reflex.

Two gluten fractions were used. The first was a filtrate of the peptic-tryptic digest of gluten after protein had been denatured by autoclaving (fraction III A.F.). The second was an ultrafiltrate of an aqueous extract of gluten (fraction G.U.F.).

METHODS

Antagonism to Drug-induced Contractures in the Guinea-pig Gut.—This was tested by conventional methods using the isolated guinea-pig ileum preparation.

Twitch Response of the Guinea-pig Jejunum Stimulated Electrically.—The method of Paton (1957) was used.

Assay of Acetylcholine Output.—This was carried out on isolated terminal ileum of the guinea-pig set up in Tyrode solution containing neostigmine and morphine as described by Paton (1957).

Assay of Acetylcholine Synthesis by Guinea-pig Small Intestine.—The incubation system used was that of Feldberg and Lin (1949) and acetylcholine was assayed on the terminal ileum preparation of Paton (1957).

Preparation of Gluten Fractions.—The fractions were prepared by the methods described by Schneider, Bishop, and Shaw (1960).

RESULTS

The Effect of the Two Gluten Fractions on Contractions of the Longitudinal Muscle of Isolated Guinea-pig Intestine Induced by Acetylcholine and Substance P.—Both gluten fractions were used in the amounts which had been found to abolish the peristalic reflex of the isolated

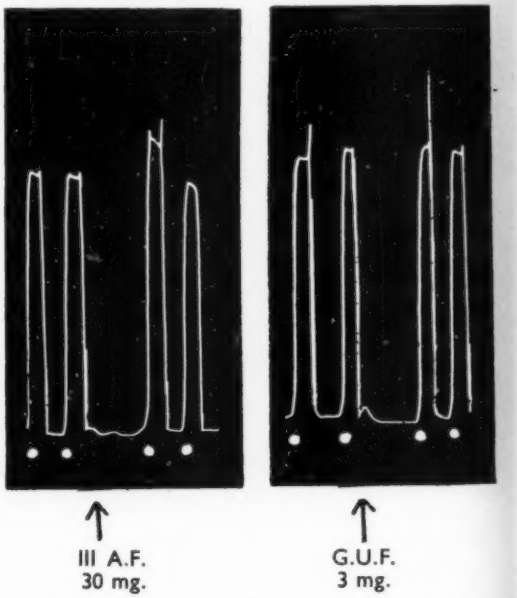

FIG. 1.—Effect of the two gluten fractions on the acetylcholine induced spasm of guinea-pig ileum. Bath volume, 15 ml.

rat jejunum in the earlier studies of Schneider, Bishop and Shaw (1960), namely, for III A.F., 2.0 mg./ml., and G.U.F., 0.2 mg./ml. A 2 min. cycle was used, and the intestine was in contact with the gluten fraction for 30 sec. There was no antagonism of the spasm induced by acetylcholine (Fig. 1), nor to that induced by substance P (Fig. 2). In both instances, the spasms in the presence of the gluten fractions were often slightly larger than those in control. The failure of the two gluten fractions to depress the responses either to acetylcholine or to substance P excluded both the possibility of a specific antagonism to either substance or of a general paralysing effect on intestinal muscle.

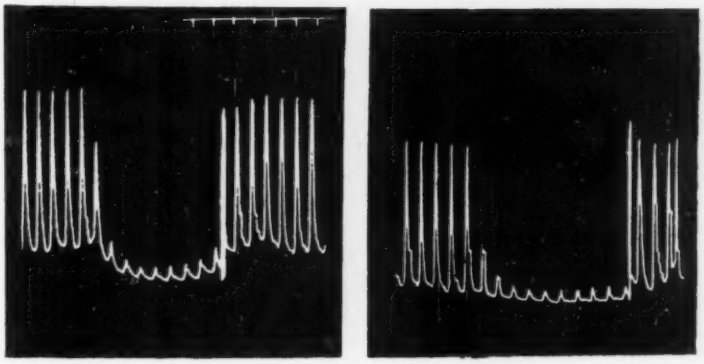

FIG. 3.—Effect of the two gluten fractions on the twitch response of coaxially stimulated guinea-pig ileum. Bath volume, 50 ml. Time, 10 sec.

The Effect of the Two Gluten Fractions on the Contraction of the Isolated Guinea-pig Jejunum Stimulated Coaxially.—Paton (1957) has shown that this preparation was sensitive to the blocking action of atropine, but was resistant to ganglionic blocking agents. Thus any depression of the response to electrical stimulation could be attributed to effects at the peripheral cholinergic nerve endings. Both gluten fractions caused a marked depression of the twitch response (Fig. 3). Usually 0.8 mg./ml. of III A.F. and 80 μg./ml. of G.U.F. decreased the response compared with 2.0 mg./ml. and 0.2 mg./ml. respectively for the peristaltic reflex. This preparation proved to be about two or three times more sensitive to the action of the gluten fractions than did the peristaltic reflex of the isolated rat jejunum.

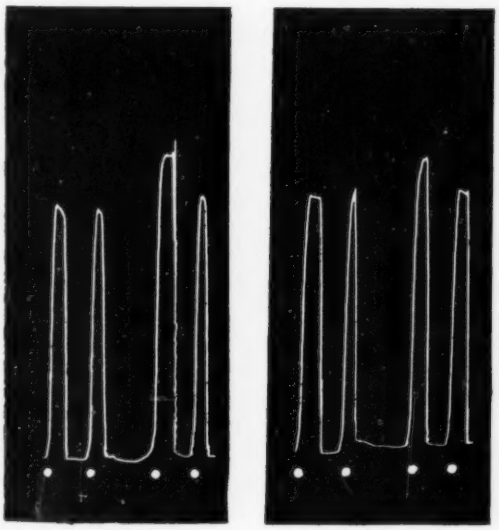

FIG. 2.—Effect of the two gluten fractions on the substance P induced spasm of guinea-pig ileum. Bath volume, 15 ml.

The Effect of the Two Gluten Fractions on the Output of Acetylcholine from the Isolated Guinea-pig Jejunum.—Results are summarized in Table I. Both gluten fractions decreased the output of acetylcholine significantly. This occurred

TABLE I

ACETYLCHOLINE OUTPUT FROM GUINEA-PIG INTESTINE

Gluten Fraction	No. of Expts.	Mean Acetylcholine Output (ng./min.)		Difference of Means (ng.)	*P*
		Control	Test		
III A.F. 40 mg./ml.					
Rest. ..	13	16·8	10·0	6·8	0·001
Stim. ..	13	30·3	17·8	12·5	0·01–0·001
G.U.F. 4 mg./ml.					
Rest. ..	11	18·6	10·9	7·7	0·01–0·001
Stim. ..	13	26·0	15·5	10·5	0·001

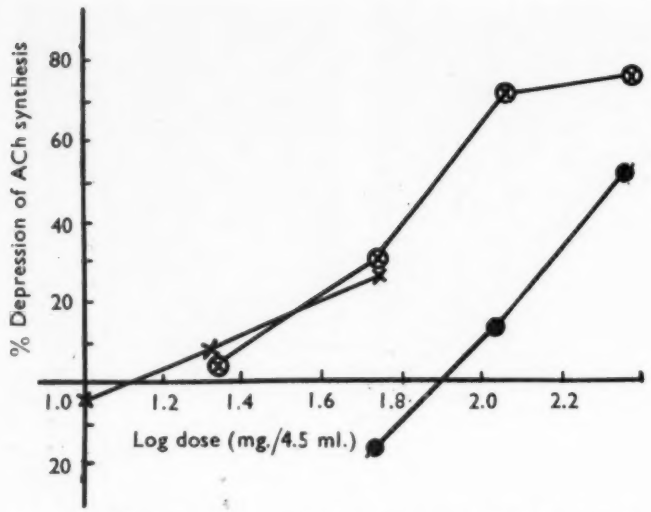

FIG. 4.—Effect of the two gluten fractions. G.U.F. X—X, III A.F. ⊗—⊗, and the peptic-tryptic digest of gelatin, Gel. III A.F. ●—●, on acetylcholine synthesis in guinea-pig small intestine.

in the resting state as well as during stimulation at 50 pulses/min. for 5 min. The ultrafiltrate G.U.F. was again about 10 times more potent than the peptic-tryptic digest III A.F.

The Effect of the Two Gluten Fractions on the Synthesis of Acetylcholine.—Addition of either of the gluten fractions to the incubation system caused a decrease in acetylcholine synthesis which became more marked with higher doses (Fig. 4). When the amount of III A.F. was raised from 4.4 to 11 mg./ml., the decrease in synthesis rose from 4 to 27% and reached 70% when 22 mg./ml. was used. Amounts of G.U.F. of 22 mg./ml. and over could not be tested, for they were spasmogenic. With the lower doses the curves for the two gluten fractions relating dose to decrease in acetylcholine synthesis were superimposable. In order to determine whether this depression of synthesis was a characteristic feature of gluten fractions, or whether it was a non-specific effect, experiments were carried out with a peptic-tryptic digest of gelatin (Gel. III A.F.) which had no action in depressing the output of acetylcholine from the isolated guinea-pig jejunum. Depression of synthesis was again found, but the gelatin digest was less potent than the corresponding gluten fraction (Fig. 4).

DISCUSSION

There are several ways in which the two gluten fractions might depress the peristaltic reflex. (1) Complete paralysis of both longitudinal and circular muscle could abolish the reflex. This mechanism may be excluded, for, in the presence of the gluten fractions, the longitudinal muscle of the intestine remained sensitive to acetylcholine and substance P. (2) Ganglionic blockade could depress the peristaltic reflex, but this mechanism was unlikely as the two gluten fractions depressed the twitch response of the guinea-pig jejunum to coaxial stimulation and this preparation is insensitive to the action of ganglionic blocking agents (Paton, 1957). (3) Antagonism to a substance occurring physiologically and which stimulates smooth muscle directly such as acetylcholine or possibly substance P might have caused depression. This possibility was excluded, for the gluten fractions did not antagonize the effects of either. Using the rat fundus preparation of Vane (1957), antagonism to 5-hydroxytryptamine could not be demonstrated as both gluten fractions caused a spasm which would have masked any possible depressant effect (Schneider and Bishop, unpublished observation). Antagonism to 5-hydroxytryptamine, however, is unlikely, as severe 5-hydroxytryptamine depletion does not abolish the peristaltic reflex (Bülbring and Crema, 1959). (4) The remaining possible mode of action was by depression of acetylcholine production at cholinergic nerve endings as has been claimed for morphine (Schaumann, 1957, and Paton, 1957). In fact the acetylcholine output from the resting as well as by the electrically stimulated isolated guinea-pig jejunum was decreased by both gluten fractions. The clinical observations of Ingelfinger and Moss (1942) are in accord with our findings. Using an intraluminar balloon technique, these workers found depression of small intestinal motility and tone in two cases of "sprue." Function of the small intestine was restored by the administration of methacholine but not by the anticholinesterase, neostigmine. The authors concluded that, in the syndrome, there was an inability of the intestinal autonomic nervous system to liberate acetylcholine.

Such depression of acetylcholine production could be due either to depressed synthesis or depressed liberation from its inactive precursor. Experiments on the acetylcholine synthesizing system in the guinea-pig small intestine showed that the addition of either gluten fraction could depress synthesis and suggested that there was a relationship between dose and effect. However, with the doses which decreased acetylcholine

output (4.0 mg./ml. for III A.F. and 0.4 mg./ml. for G.U.F.) there was no depression of acetylcholine synthesis. With larger doses (11 mg./ml.) both gluten fractions were equally potent in depressing synthesis, even though G.U.F. was at least 10 times more potent than III A.F. in decreasing acetylcholine output. As this depression of acetylcholine synthesis might be a non-specific effect of peptides due to the large amount of material used, the peptic-tryptic digest of gelatin (Gel III A.F.) was tested and found in large amounts (22 mg./ml.), to depress acetylcholine synthesis. It was, however, less potent than fraction III A.F. From these experiments it was concluded that both mechanisms might contribute to the depression of acetylcholine output by gluten fractions, but that depression of liberation of acetylcholine was more important than depression of synthesis.

These studies are part of a collaborative programme of work on the pathogenesis of gluten-induced enteropathy which is being undertaken in this department under the general direction of Professor A. C. Frazer. We wish to thank Mrs. B. Matthews for technical assistance and the Energen Foods Co. for supplies of gluten.

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TREMOR IN CATS: THE EFFECT OF ADMINISTRATION OF DRUGS INTO THE CEREBRAL VENTRICLES

BY

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In cats, tremor produced by intraperitoneal pentobarbitone sodium or by intramuscular chlorpromazine was abolished by injection into the cerebral ventricles of a few gamma of adrenaline or noradrenaline, but not of other sympathomimetic amines or of anti-Parkinsonian drugs. Chloralose, urethane, calcium chloride, and magnesium chloride produced anti-tremor activity when administered in this way. When adrenaline or noradrenaline was perfused from a lateral ventricle to the aqueduct during pentobarbitone sodium tremor, tremor was inhibited when less than 1 μg. had been perfused. Adrenaline was at least four times as active as noradrenaline. Isoprenaline inhibited tremor when perfused in higher concentrations, but perfusion of ephedrine, amphetamine or of the anti-Parkinsonian drugs in high concentrations was ineffective. Perfusion of 2 μg. of chloralose or of 20 μg. of calcium chloride was sufficient to inhibit tremor. Intraventricular injections of 250 to 500 μg. of 5-hydroxytryptamine initiated tremor. Possible physiological implications of these findings are discussed.

The present experiments deal with the action of drugs in initiating and abolishing tremor when administered by the intraventricular route, that is, by injection into the cerebral ventricles or by perfusion from a lateral ventricle to the aqueduct. Feldberg and Malcolm (1959) observed that tubocurarine perfused in this way produced tremor in cats anaesthetized with chloralose, and that the tremor was abolished when adrenaline or noradrenaline was added to the perfusing fluid. The concentration of the amines employed in these experiments was strong, as was the concentration of tubocurarine required to produce tremor. The question therefore arose as to whether adrenaline or noradrenaline exerted anti-tremor activity, when used in more physiological concentrations, on tremor produced by other means.

Intraperitoneal pentobarbitone sodium (Hall and Goldstone, 1940; Lippold, Redfearn and Vuco, 1959) and intramuscular chlorpromazine (Kaelber and Joynt, 1956) produce tremor in cats, and Hall and Goldstone have shown that the pentobarbitone sodium tremor is abolished by an intravenous infusion of adrenaline, 4 μg./kg. per min.; an intravenous injection of 80–150 μg./kg. first accentuated and then abolished tremor. It has now been found that both the pentobarbitone sodium and the chlorpromazine tremors are abolished by small amounts of adrenaline or noradrenaline administered by the intraventricular route. Of other sympathomimetic amines examined in this way, isoprenaline was found to have anti-tremor activity but only when administered in much larger amounts than adrenaline or noradrenaline.

Another well-known action of intraventricular adrenaline and noradrenaline is sedation, or an anaesthesia-like condition (for references see Feldberg, 1958). A similar effect occurs with intraventricular chloralose, urethane, calcium and magnesium (Feldberg and Sherwood, 1957; Feldberg, 1958). These substances were therefore also examined by the intraventricular route, and found to inhibit the pentobarbitone sodium and chlorpromazine tremor. On the other hand, anti-Parkinsonian drugs administered in this way did not affect these tremors. Finally, while investigating the effect of intraventricular 5-hydroxytryptamine, the substance itself was found to initiate tremor.

METHODS

The experiments were carried out on cats. For the injection of drugs into the cerebral ventricles a Collison cannula was implanted aseptically into the left lateral ventricle during pentobarbitone sodium anaesthesia as described by Feldberg and Sherwood (1953). After recovery from the operation the cats

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were used for experiments with intraventricular injections once or twice a week. The drugs were injected intraventricularly in a volume of 0.25 ml.

Perfusion from Lateral Cerebral Ventricle to Aqueduct.—The cats were anaesthetized with intraperitoneal pentobarbitone sodium (30 mg./kg.). The trachea was cannulated and, with the cat lying on its back, the head was fixed in a raised and flexed position so that the surface of the skull was horizontal to the ear bars and mouth piece of a head holder, similar to the Horsley Clark stereotaxic instrument. The method of perfusion was that described by Bhattacharya and Feldberg (1958). The inflow was through a Collison cannula implanted into the left lateral ventricle, the outflow through a polythene tube inserted through the opened cisterna magna, so that the tip of the cannula lay in the middle of the aqueduct. This fact was ascertained at the end of several experiments by perfusing the dye bromophenol blue and then making a sagittal section through the brain. The perfusion fluid was the artificial cerebrospinal fluid described by Merlis (1940) but without the bicarbonate. The bicarbonate was replaced by an equivalent amount of NaCl in order to diminish the destruction of adrenaline and noradrenaline in the alkaline fluid. The composition of the perfusion fluid was as follows (g./l.): NaCl 8.98; KCl 0.25; $CaCl_2$ 0.14; $MgCl_2$ 0.11; NaH_2PO_4 0.07; urea 0.13; and glucose 0.61.

Perfusion was maintained by a continuous slow injector (C. F. Palmer) at a rate of 0.1 ml./min. The fluid in the syringe of the injector passed by means of polythene tubing first through a small air chamber where the inflow could be checked, then through a water jacket in which water at 38° was circulating by means of an air lift, and finally through the shaft of a fine syringe needle which was inserted through the rubber diaphragm closing the top of the Collison cannula. The substances to be perfused were dissolved in the artificial cerebrospinal fluid and a second continuous slow injector was filled with the solution. The switch-over of perfusion from one injector to the other was done by withdrawing the needle of the one and inserting that of the other in its place; this took less than a second.

Recording of Tremor.—In cats anaesthetized with pentobarbitone sodium records of tremor were obtained from the hind legs by attaching the paw or the tendon of the tibialis anterior muscle by means of a thread to a light tension lever, the movements of which were traced on a smoked drum.

Substances Applied by the Intraventricular Route.—The following substances were injected into or perfused through the cerebral ventricles: adrenaline bitartrate; noradrenaline bitartrate; 5-hydroxytryptamine creatinine sulphate; creatinine sulphate; amphetamine sulphate; ephedrine sulphate; phenylephrine hydrochloride (Neosynephrine); isoprenaline sulphate; atropine sulphate; hyoscine hydrochloride; benzhexol (Artane); diethazine hydrochloride (Diparcol); chloralose; urethane; calcium chloride and magnesium chloride. When the substances used were salts, the doses given in the text refer to the salts.

RESULTS

Chlorpromazine Tremor

Intramuscular injection of 25 mg./kg. chlorpromazine hydrochloride produced tremor, but whereas relaxation of the pupils, ataxia and sedation occurred within a few minutes, tremor did not begin until 1 to 4.5 hr. after the injection. It then continued sometimes for several hours and was most clearly observed when the animal was lying on its side. Chlorpromazine tremor is known to be related to hypothermia, which in its turn depends on the environmental temperature. This, in the present experiments, was between 19 and 23°.

Sympathomimetic Amines. — Intraventricular injection of 5 μg. of either adrenaline or noradrenaline stopped tremor for 10 to 25 min. In some cats the intraventricular injection of 2.5 μg. of either amine decreased or stopped tremor for a few minutes, but in others this dose was ineffective. Intraventricular injections of amphetamine or phenylephrine tested in doses of up to 500 μg. did not affect tremor.

Chloralose.—Intraventricular injection of 0.5 mg. chloralose stopped tremor for 1 to 2 hr.

Calcium Chloride.—Intraventricular injection of 0.5 mg. calcium chloride stopped tremor for 10 to 20 min.; an injection of 0.25 mg. or less was ineffective.

Anti-Parkinsonian Drugs.—Atropine, hyoscine and benzhexol injected intraventricularly in amounts of up to 0.5 mg. did not affect tremor.

Pentobarbitone Sodium Tremor

Intraperitoneal injection of 30 mg./kg. pentobarbitone sodium initiated a fine fast tremor or shivering 30 to 150 min. after the injection. Tremor continued for at least 1 hr. and sometimes for as long as 5 hr. When the cat was lying on its side, tremor usually began in the flank, gradually spread over the hind limbs and later involved the whole body, including the neck and the head. Often the intensity of tremor increased during this period. In different cats the intensity of tremor showed pronounced variations, but in the same cat tremor did not vary, or varied only a little, when the pentobarbitone sodium was injected on different days. In several experiments the rate of tremor recorded on a fast-moving smoked drum was found to vary between 10 and 18/sec.

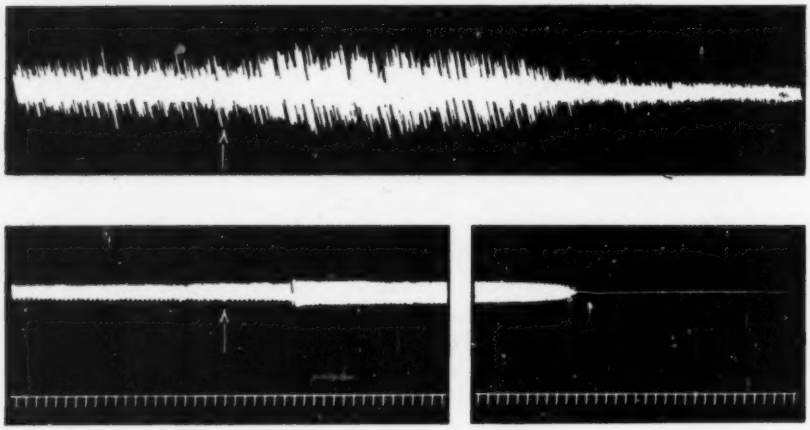

FIG. 1.—Tremor recorded from left hind leg of a cat anaesthetized with pentobarbitone sodium. At the arrows intraperitoneal injections of pentobarbitone sodium (9 mg./kg.). Lower record taken approximately 0.5 hr. after upper record. The interval between the two sections of the lower record was 5 min. Time signals in 10 sec.

When during the recovery phase from the anaesthesia the cat sneezed, or spontaneous movements occurred, tremor ceased but returned once the movement was completed.

In most cats the onset of tremor occurred when the rectal temperature had fallen to between 35.5 and 36.5°. During tremor the temperature rose, but tremor continued for some time after the temperature had returned to the normal level, or had reached a level above normal, that is, 41°. In a few cats tremor occurred without a fall in rectal temperature, or with a slight fall only, so that in these there was no correlation between tremor and fall in rectal temperature.

When additional pentobarbitone was injected intraperitoneally during the period of tremor it caused diminution or cessation of tremor. This was often preceded by a short period of increased strength of tremor. An example of this is shown in Fig. 1. Intraperitoneal injection of 9 mg./kg. pentobarbitone sodium caused first an increase and then a decrease in the amplitude of tremor, but no cessation (upper record). A subsequent injection caused only slight initial accentuation of tremor and then cessation (lower record).

Effects of Intraventricular Injections of Drugs on Pentobarbitone Sodium Tremor

Sympathomimetic Amines. — Intraventricular injection of adrenaline or noradrenaline abolished tremor. In some cats a dose of 2.5 μg. abolished tremor, in others it caused attenuation and in still others it had no effect. Most cats responded to 5 μg. either with abolition or with diminution of tremor and 10 μg. always abolished it. Following the injection, tremor either ceased abruptly within 1 or 2 sec. or disappeared gradually within 30 to 90 sec. After 5 μg. tremor returned within 10 to 20 min., and after 10 μg. within 20 to 35 min.

Intraventricular injections of ephedrine, amphetamine, isoprenaline or phenylephrine, in doses ranging from 5 to 500 μg., did not affect the tremor.

Chloralose, Urethane.—Intraventricular injection of 0.25 mg. of chloralose decreased or abolished tremor for about 20 min.; 0.5 mg. abolished tremor for as long as 2 hr. Urethane had transient anti-tremor effect when injected intraventricularly in large doses. Less than 10 mg. was ineffective whereas 20 to 30 mg. decreased or stopped tremor for 3 to 6 min.

Calcium Chloride.—Intraventricular injection of 0.35 to 0.5 mg. decreased or stopped tremor for 20 to 50 min. Such an experiment is illustrated in Fig. 2.

Magnesium Chloride.—Intraventricular injection of 0.5 mg. decreased tremor for about 10 min. The effect began after a latency of about 10 min. After an injection of 1 or 2 mg. the latency was shorter and tremor decreased or ceased for 30 to 50 min. In the experiment, shown in Fig. 3, the injection of first 1 mg. and then 2 mg. decreased the amplitude of the temor but did not abolish it. In another experiment these doses of magnesium chloride caused cessation of tremor.

Anti-Parkinsonian Drugs. — Intraventricular injections of atropine, hyoscine, diethazine and trihexiphenidyl in doses ranging from 5 to 500 μg. did not affect the tremor. With atropine and hyoscine it was further found that they did not affect the responses to subsequent intraventricular injections of adrenaline or noradrenaline.

Effects of Drugs Perfused from Lateral Ventricle to Aqueduct on Pentobarbitone Sodium Tremor

Sympathomimetic Amines.—On perfusion the anti-tremor effect of adrenaline or noradrenaline

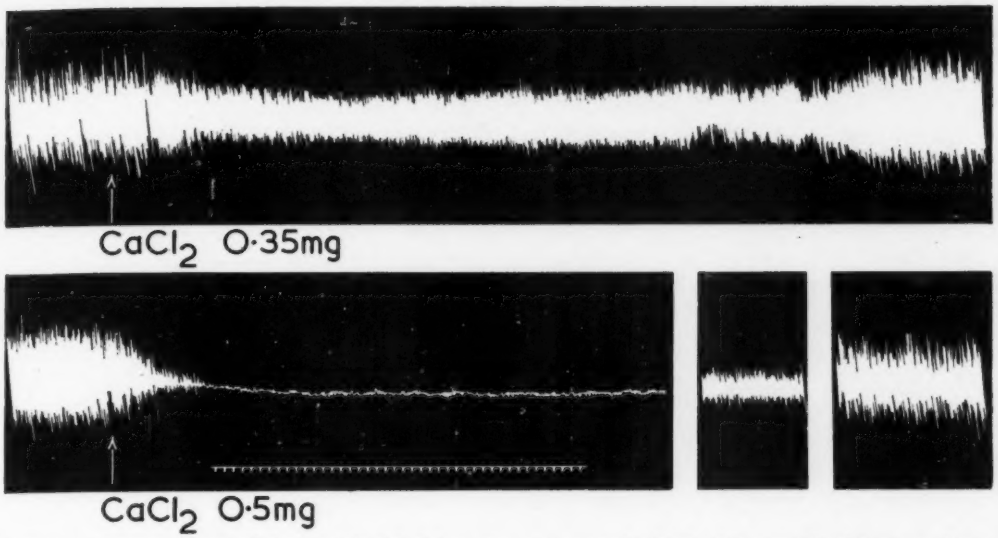

FIG. 2.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. At the arrows intraventricular injection of 0.35 mg. (upper record) and of 0.5 mg. (lower record) of calcium chloride. In the lower record there was an interval of approximately 0.5 hr. between each section of the record. Time signals in 10 sec.

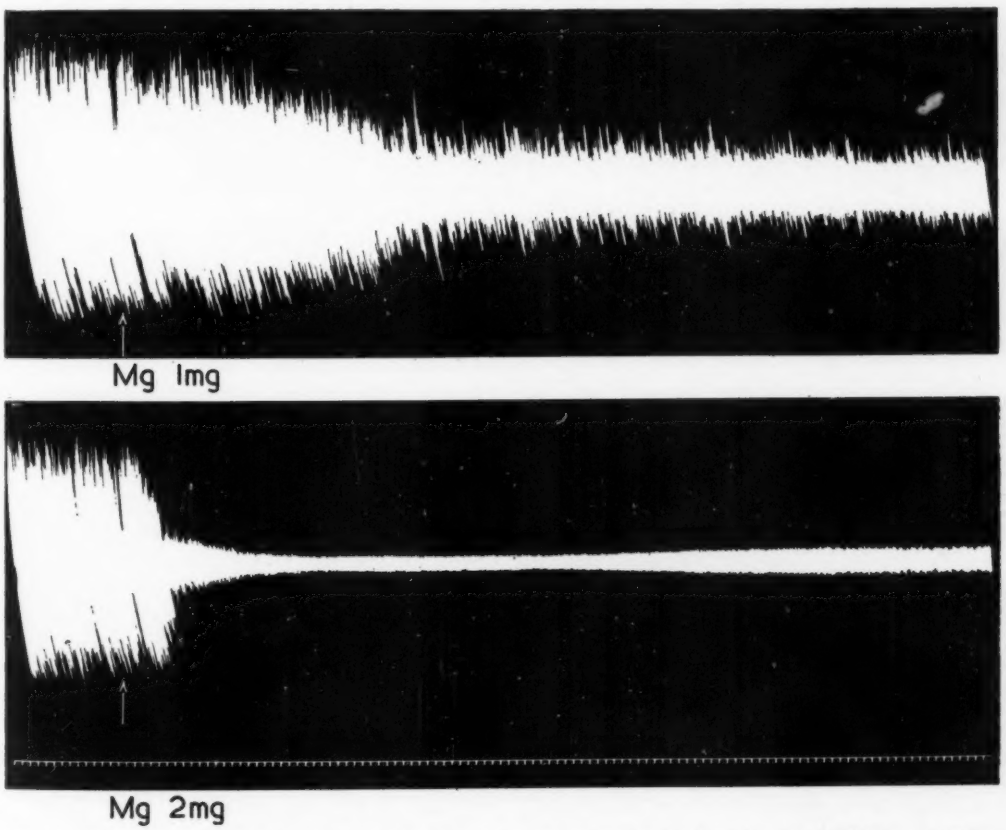

FIG. 3.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. At arrows intraventricular injections of 1 mg. (upper record) and 2 mg. (lower record) of magnesium chloride. The 2 mg. was injected 40 min. after the 1 mg. Time signals in 10 sec.

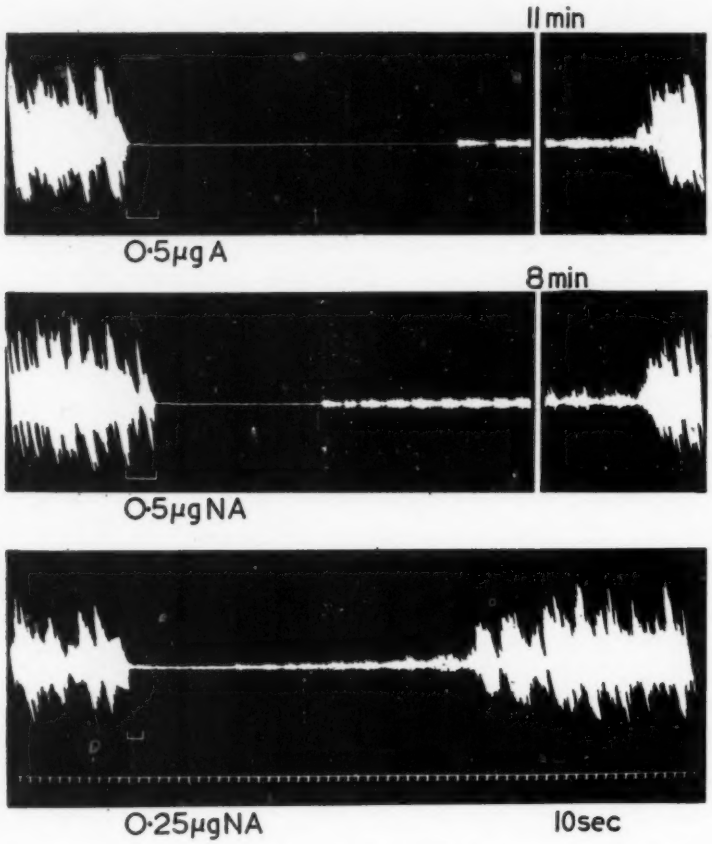

FIG. 4.—Tremor recorded from left tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. At the signals perfusion was with adrenaline (A) or noradrenaline (NA) 1/100,000 from lateral ventricle to aqueduct at a rate of 0.1 ml./min. for 30 sec. (0.5 μg.) or for 15 sec. (0.25 μg.). In the upper two records the intervals between the two sections were 11 and 8 min. respectively. Time signals in 10 sec.

was obtained with even smaller doses than those effective on intraventricular injection. In the experiment of Fig. 4, the amines were perfused in a concentration of 1/100,000. The upper two records show cessation of tremor on perfusion for 30 sec. and the lower record on perfusion for 15 sec., that is, when 0.5 and 0.25 μg. respectively entered the cerebral cavities. As the effects were readily reproducible during succeeding periods of perfusion the relative potencies of the two amines could easily be ascertained. Fig. 5 illustrates the finding that adrenaline is at least four times more potent than noradrenaline in inhibiting tremor. Perfusion with adrenaline 1/250,000 (upper record) was much more effective than a similar concentration of noradrenaline (middle record), and perfusion with adrenaline 1/1,000,000 (lower record) was slightly more effective than the perfusion with noradrenaline 1/250,000. Onset of inhibition in each case occurred within 1 min. of perfusion, that is, after 0.4 μg. of the stronger and 0.1 μg. of the weaker solution had been perfused.

Isoprenaline which on intraventricular injection did not inhibit the pentobarbitone sodium tremor in doses up to 500 μg. did so when perfused from the lateral ventricle to the aqueduct although it was less potent than adrenaline or noradrenaline. The upper record of Fig. 6 illustrates the result obtained on perfusion with isoprenaline 1/10,000. The strength of tremor was affected within 0.5 min. of perfusion, that is, after less than 5 μg. had entered the cerebral ventricles.

Amphetamine and ephedrine did not affect the tremor when perfused through the cerebral ventricles in a concentration as strong as 1/2,000. This is shown in the middle two records of Fig. 6. The lowest record serves as a control and shows that adrenaline was effective in a low concentration.

Chloralose.—As shown in Fig. 7, perfusion with chloralose 1/50,000 for 10 min. abolished tremor. The effect began after 1 min. perfusion, that is, after 2 μg. chloralose had entered the cerebral ventricles, and persisted for nearly 10 min. after the perfusion solution was changed back to artificial cerebrospinal fluid. On perfusion with stronger solutions of chloralose the effect lasted longer.

Calcium Chloride.—Perfusion in a concentration of 1/100,000 calcium chloride decreased, and of 1/50,000 abolished, tremor. The effect began after about 2 min. perfusion, that is, after 20 and 40 μg. respectively had entered the cerebral ventricles.

Anti-Parkinsonian Drugs.—Atropine, hyoscine, trihexiphenidyl and diethazine did not affect tremor when perfused from the lateral ventricle to the aqueduct in a concentration as strong as 1/2,000.

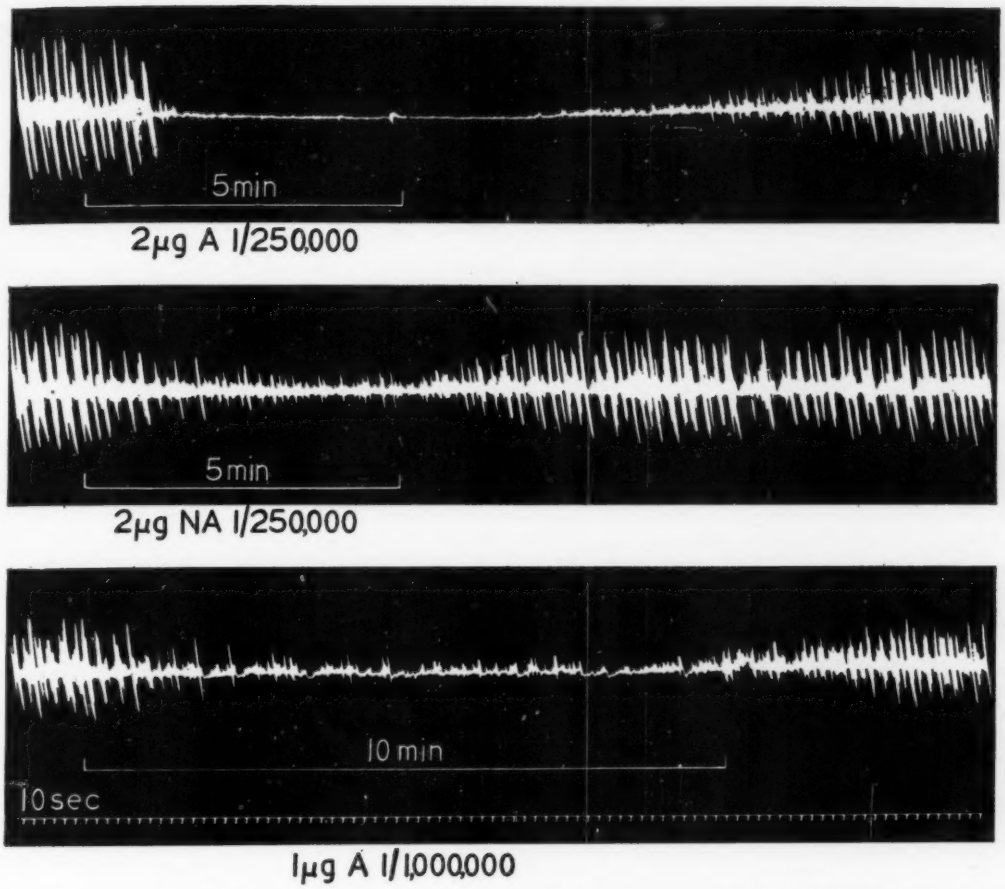

FIG. 5.—Tremor recorded from left tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. At the signals perfusion was with adrenaline (A) or noradrenaline (NA) from lateral ventricle to aqueduct. Upper two records: perfusion of the amines in a concentration of 1/250,000 for 5 min. at a rate of 0.1 ml./min. (2 μg.). Lower record: perfusion of adrenaline in a concentration of 1/1,000,000 for 10 min. at a rate of 0.1 ml./min. (1 μg.). Time signals in 10 sec.

5-Hydroxytryptamine Tremor

In non-anaesthetized conscious cats intraventricular injections of 500 μg. 5-hydroxytryptamine produced tremor which started within 1 min. or 2 min. after the injection. Usually it appeared first in the ears, then the hind legs became involved and finally there was tremor over the whole body. Tremor was continuous, but sometimes bursts of greater amplitude occurred. Tremor lasted from 20 min. to over 2 hr. At the height of activity, tremor could be seen in the legs of the standing cat and the entire head shook due to the strength of the tremor. Intraventricular injection of 250 μg. was also effective; tremor lasted for 10 to 20 min. During, and following, the period of tremor the cat was usually less alert than before the injection and would be sitting or lying in the cage with its head erect. There was a tendency for the eyelids to close. The ears were hot due to dilatation of their vessels.

During the initial stage of a pentobarbitone sodium anaesthesia before the pentobarbitone sodium induced tremor occurred, tremor could be initiated by intraventricular 5-hydroxytryptamine and this tremor was recorded. Such an experiment is illustrated in Fig. 8. It shows the sudden onset of tremor and its rate when recording was on a fast-moving drum. The rate was approximately 17/sec.

Creatinine sulphate itself injected intraventricularly in equivalent amounts did not initiate tremor.

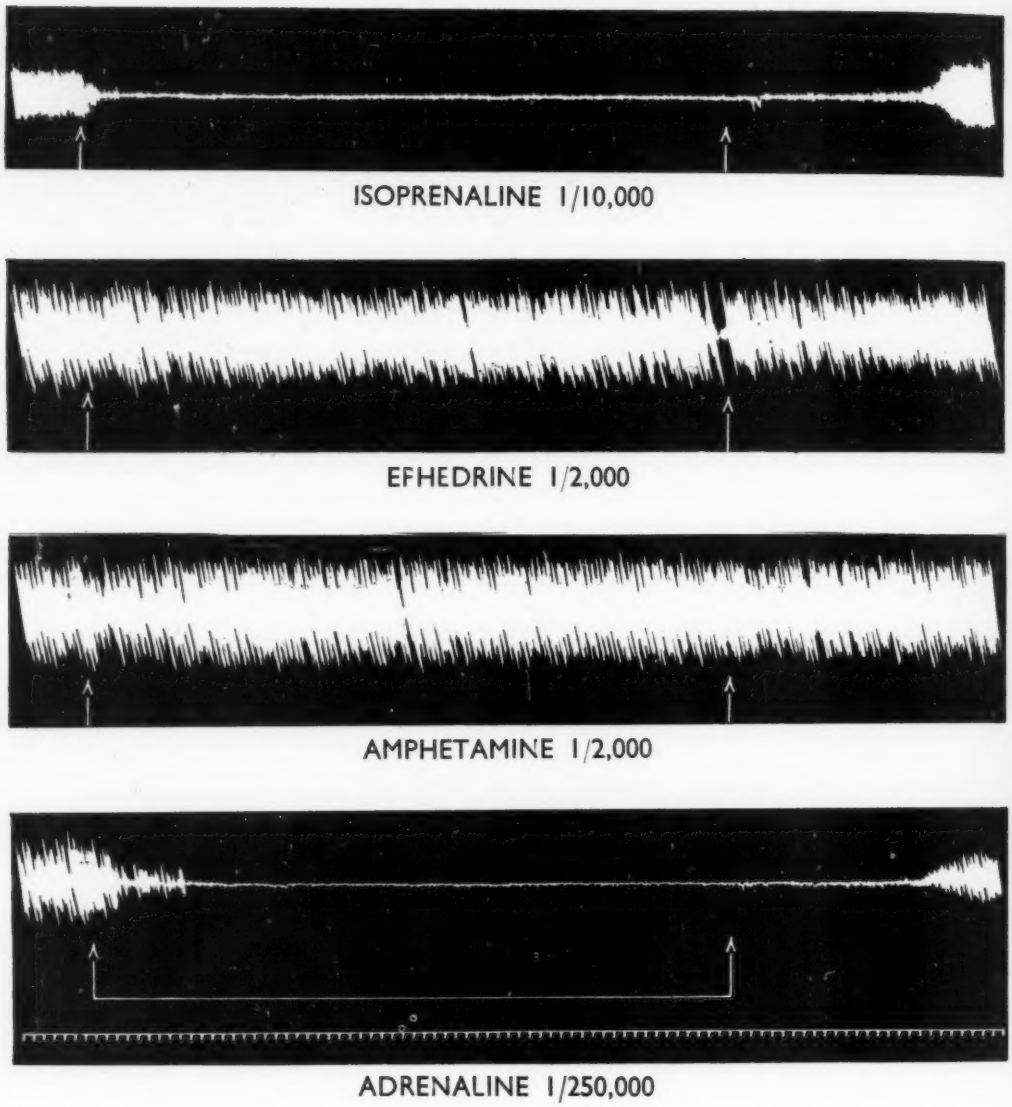

FIG. 6.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. Between the arrows perfusion was with isoprenaline 1/10,000 (upper record), ephedrine 1/2,000 (second record from top), amphetamine 1/2,000 (third record from top) and adrenaline 1/250,000 (lowest record), from lateral ventricle to aqueduct for 10 min. at a rate of 0.1 ml./min. Time signals in 10 sec.

DISCUSSION

No distinction has been made when describing the results between tremor and shivering. Lippold, Redfearn and Vučo (1959) looked upon the rhythmical muscular activity which occurred in cats anaesthetized with pentobarbitone sodium as shivering, and likened it to the shivering which occurs in man exposed to cold. Hall and Goldstone (1940) combined pentobarbitone sodium anaesthesia with submersion of the cat in cold water in order to produce shivering. In most of our experiments the onset of tremor occurred when the rectal temperature of the cat had fallen during the pentobarbitone sodium anaesthesia to between 35.5 and 36.5°, but tremor continued after the temperature had returned to normal or

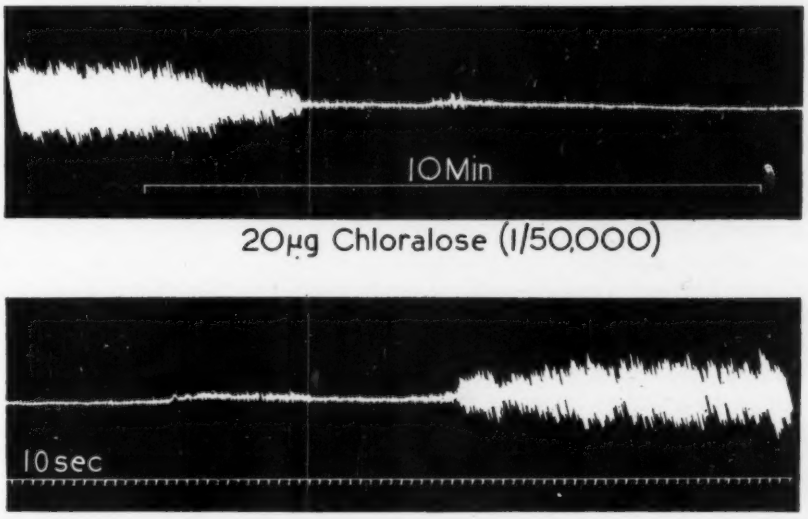

FIG. 7.—Tremor recorded from right tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. At the signal perfusion was with chloralose 1/50,000 from lateral ventricle to aqueduct for 10 min. at a rate of 0.1 ml./min. (20 µg.). Lower record continuation of upper record. Time signals in 10 sec.

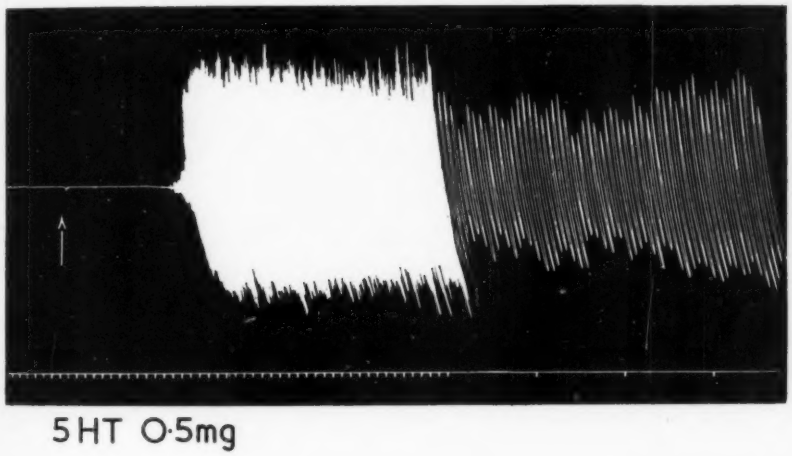

FIG. 8.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. At the arrow intraventricular injection of 0.5 mg. 5-hydroxytryptamine. Left half of record taken on a slow-moving drum: time signals in 10 sec. Right half of record taken on a fast-moving drum: time signals in 1 sec.

above normal level. In some cats tremor began without any change in rectal temperature. We know that rectal temperature does not necessarily reflect the temperature of the blood passing through the brain. The absence of a correlation between rectal temperature and onset of tremor therefore does not exclude the lowering of blood temperature as the cause of the pentobarbitone sodium tremor. Yet the transient increase in the strength of tremor observed on deepening the anaesthesia by additional pentobarbitone sodium, injected intraperitoneally, would be difficult to explain on the assumption that the pentobarbitone sodium tremor is solely dependent on a fall in temperature. The tremors initiated by cold or by anaesthesia, though not dependent on each other, may nevertheless be essentially the same phenomenon. In this connexion, it is interesting to note that adrenaline, which has such a strong action on the pentobarbitone sodium tremor, also affects shivering produced by cold. Tournade, Raynaud and Chardon (1939) found that intravenous adrenaline decreases shivering in dogs exposed to cold.

Since chlorpromazine causes depression of body temperature (Eriksson and Wasz-Höckert, 1959), its mode of action in producing tremor is probably similar to that of pentobarbitone sodium and cold. Weight is added to this view in that the same agents which depressed pentobarbitone sodium tremor also depressed tremor produced by chlorpromazine. The fact that both types of tremor were refractory to the action of drugs employed in the treatment of Parkinsonism may mean that a different mechanism is responsible for Parkinson's tremor.

The finding that a number of substances influence tremor or shivering when given by the intraventricular route poses the question of where they act when producing this effect. It is known that tremor-like movements are produced by electrical stimulation of the medial portions of the brain stem which in monkeys reach from the mesencephalon to the medulla (Jenkner and Ward, 1953) but which in cats extend further rostrally to the hypothalamus (Birzis and Hemingway, 1957). It is reasonable to assume that the substances given intraventricularly influence tremor activity by acting on some of these structures. Those in the medulla and caudal portion of the mesencephalon can be excluded as the main site of action because the effects were also obtained when the substances were perfused from the lateral ventricle to the middle of the aqueduct. The assumption that the drugs act on the midline structures in the diencephalon and rostral part of the mesencephalon, electrical stimulation of which results in tremor-like movements, implies that they are able to penetrate into the brain substance lining the third ventricle and the aqueduct. Previous experiments in which histamine or the dye bromophenol blue was perfused from the lateral ventricle to the aqueduct showed that these substances penetrate into the grey matter surrounding these cavities (Draskoci, Feldberg, Fleischhauer and Haranath, 1960; Feldberg and Fleischhauer, 1960) and it is likely that other substances penetrate in a similar way.

Whenever a substance artificially administered acts in minute amounts on structures in which it is usually found, one is led to consider the possibility that the observed pharmacological effect mimics a physiological function. Fractions of a microgram of either adrenaline or noradrenaline when perfused from the lateral ventricle to the aqueduct were sufficient to suppress tremor. Vogt (1954) has shown that the highest concentrations of noradrenaline occur in the midline structures of diencephalon and mesencephalon. The concentration of noradrenaline in the perfusion fluid necessary to produce anti-tremor activity approximates that found in these mid-brain structures, but the concentration attained must be smaller than in the perfusion fluid itself due to dilution during its passage to the effector sites. Could it be that the physiological release of the brain stem noradrenaline is involved in the control of tremor and shivering? At present this question cannot be answered because the relevant physiological experiments have not been performed. However, the results obtained with minute doses of noradrenaline perfused from the lateral ventricle to the aqueduct at least point in this direction. The same question is applicable to the brain stem adrenaline. Although it contributes only a small proportion of the brain stem sympathin (Vogt, 1954) our finding that it is at least four times more potent than noradrenaline in anti-tremor activity is compatible with the view that adrenaline shares in this central nervous function.

It appears that the structure of the sympathomimetic amines essential for anti-tremor activity is the catechol ring, since it was found that only those having this moiety, that is, adrenaline, noradrenaline and isoprenaline, possessed this action.

The anti-tremor activity of adrenaline and noradrenaline was shared by a number of substances which, like these amines, produce sedation or an anaesthesia-like condition on intraventricular injection, suggesting a relationship between the central depression which leads to anaesthesia and anti-tremor activity. This suggestion is supported by the finding that deepening of the pentobarbitone sodium anaesthesia by additional pentobarbitone sodium into a cat during the period of tremor often increased its amplitude for a short time before tremor decreased and finally ceased. This would imply that before the injection the cat was above the level of anaesthesia at which maximal strength of tremor is exhibited and that it passed through this level as the anaesthesia deepened.

Noradrenaline and adrenaline are not the sole amines which occur normally in the central nervous system. Amin, Crawford and Gaddum (1954) showed that 5-hydroxytryptamine has a similar distribution in the brain stem although the absolute quantities are smaller than those of noradrenaline. It has now been found that 5-hydroxytryptamine initiates tremor on intraventricular injection. This amine may, therefore, also be involved in regulation of tremor activity. The amounts necessary to produce the effect, however, were large. They were much greater than those of noradrenaline or adrenaline required to suppress tremor. This may be due to the remoteness of the site of action of 5-hydroxytryptamine from the ventricular cavity and aqueduct which would entail more dilution due to the greater distance traversed. If the brain stem 5-hydroxytryptamine were to exert a tremor inducing function, the higher concentration of the anti-tremor amine, noradrenaline, in this region may serve to prevent a continuous state of tremor.

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TRANSMISSION FAILURE IN SYMPATHETIC NERVES PRODUCED BY HEMICHOLINIUM

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It has been shown by others that hemicholinium (α,α'-dimethylethanolamino-4,4'-biacetophenone) inhibits the synthesis of acetylcholine, an effect which is reversed by choline. Hemicholinium produces a failure of response to nerve stimulation in the following sympathetically innervated preparations: guinea-pig isolated vas deferens, rabbit isolated uterus, rabbit isolated colon, perfused rabbit ear, cat isolated atria and the piloerector muscles in the cat's tail. The blocking action of hemicholinium on the responses to postganglionic sympathetic stimulation resembles its blocking action against cholinergic nerve stimulation observed on rabbit isolated atria with vagus nerves, rabbit isolated vagina with pelvic nerves, and guinea-pig isolated diaphragm with phrenic nerve. The failure of transmission produced by hemicholinium in sympathetic nerves and in cholinergic nerves can be reversed by choline. It is suggested that if there were a cholinergic junction at sympathetic nerve endings the mechanism of the blocking action of hemicholinium at these endings could be explained by inhibition of acetylcholine synthesis.

Hemicholinium (α,α'-dimethylethanolamino-4,4'-biacetophenone) produces a failure of response to cholinergic nerves (MacIntosh, Birks, and Sastry, 1956; Reitzel and Long, 1959a, b; Wilson and Long, 1959). MacIntosh *et al.* (1956) and Gardiner (1957) found that hemicholinium inhibits the synthesis of acetylcholine by nervous tissues. This observation led them to suggest that the transmission failure is caused by the loss from the nerve endings of acetylcholine which cannot be replaced by resynthesis in the presence of hemicholinium.

Rand and Chang (1960) observed that hemicholinium blocks the responses of the guinea-pig isolated vas deferens to stimulation of the sympathetic hypogastric nerve. This blocking action of hemicholinium has a number of features in common with the block of cholinergic nerves, and they suggested that the site of the block was similar and that there was a cholinergic mechanism involved in sympathetic nerves.

We have now carried out further investigations on the effects of hemicholinium on the responses of a number of organs to nerve stimulation.

METHODS

Sympathetically Innervated Preparations. — The guinea-pig vas deferens with hypogastric nerve attached was prepared by the method developed by Huković (described by Huković, 1960b, and Boyd, Chang, and Rand, 1960); the rabbit uterus with hypogastric nerve by the method of Varagić (1956); the rabbit colon with lumbar sympathetic nerve as described by Finkleman (1930). The nerves were passed through a tube containing bipolar platinum electrodes of the type described by Burn and Rand (1960a). These organs were suspended in McEwen (1956) solution or in Krebs' bicarbonate solution bubbled with 95% oxygen and 5% carbon dioxide in a 50 ml. bath at 30°.

Experiments on the sympathetically innervated perfused rabbit ear were carried out by the method of Gaddum and Kwiatkowski (1938). The superior cervical ganglion was stimulated with bipolar platinum electrodes; one was placed distal to the ganglion, the other on the ganglion. The outflow of drops of perfusion fluid from the ear was measured with a Gaddum or a Thorp recorder (C. F. Palmer, London).

Observations were made with isolated sympathetically innervated atria of the cat heart. Huković (1959) has described the preparation of isolated sympathetically innervated rabbit atria in which he stimulated the cervical sympathetic trunk close to the stellate ganglion. In order to ensure that the sympathetic stimulation was postganglionic we used a different procedure. Anaesthesia was induced with ether in small cats (1 to 2 kg.) and continued with intravenous urethane. The trachea was cannulated and artificial respiration given. The chest was opened in the midline and retracted back on the right side. The right stellate ganglion was exposed and ligatures tied around the cervical sympathetic nerve near to its

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intersection with the subclavian artery, around the thoracic sympathetic chain in the second intercostal space and around the branches of the stellate ganglion which entered the brachial plexus. The tissue between these ligatures, which included the stellate ganglion, was separated from the right posterior surface of the pleural cavity. The cat was bled out through a carotid cannula. The heart was lifted at the apex and cut away together with the lungs, trachea, oesophagus, and the muscles as close as possible to the underlying vertebral column and ribs. These tissues were transferred to oxygenated McEwen solution at 20°. In further dissection the lungs, pericardium, oesophagus, and ventricles were cut away. The atria were suspended in a 100 ml. bath of McEwen solution at 27°. The stellate ganglion was stimulated with bipolar platinum electrodes.

Observations on the pilomotor response to sympathetic stimulation were carried out as described by Burn and Rand (1960b).

Parasympathetically Innervated Preparations.—Rabbit atria with intact vagus nerves were prepared by the method of Burn and Rand (1957) and suspended in a 100 ml. bath of McEwen solution at 27°.

Some observations were made on the parasympathetically innervated rabbit vagina. The vagina was separated from the rectum and at its lower end, on the posterior surface, two blood vesels with adjacent nerves were identified. The vagina was suspended in McEwen solution in a 50 ml. organ bath to record longitudinal contractions. One of the two nerves entering the posterior wall was passed through a bipolar electrode tube.

Motor Nerve.—Observations on guinea-pig hemidiaphragm with its phrenic nerve were made by the method described by Bülbring (1946) for the rat.

Details of the electrical stimulation applied to nerves are given in the Results.

Hemicholinium (α, α' dimethylethanolamino 4, 4′-biacetophenone dibromide) was a generous gift to one of us (M. J. R.) from Dr. J. P. Long, of the Department of Pharmacology, State University of Iowa, Iowa City. The amounts of hemicholinum referred to in the text are as this salt.

Results

Guinea-pig Vas Deferens with Hypogastric Nerve.—Preliminary experiments showed that after hemicholinium in concentrations of 20 to 50 μg./ml. there was a gradual decrease of the contractions of the vas deferens in response to hypogastric nerve stimulation (Rand and Chang, 1960). The record from one experiment is shown in Fig. 1. The responses to stimulation of the nerve with 2 msec. pulses at 10/sec. for 10 sec. periods in every 2 min. were completely abolished 100 min. after hemicholinium was added to the bath. Observations in a number of similar experiments indicated that the time taken for hemicholinium to produce a complete block of response was related to the frequency of stimulation of the nerve. In the experiment shown in Fig. 2 hemicholinium (30 μg./ml.) was allowed to act for 100 min. during which time the nerve was stimulated at 5/sec. for 10 sec. periods in every 2 min., which produced only a

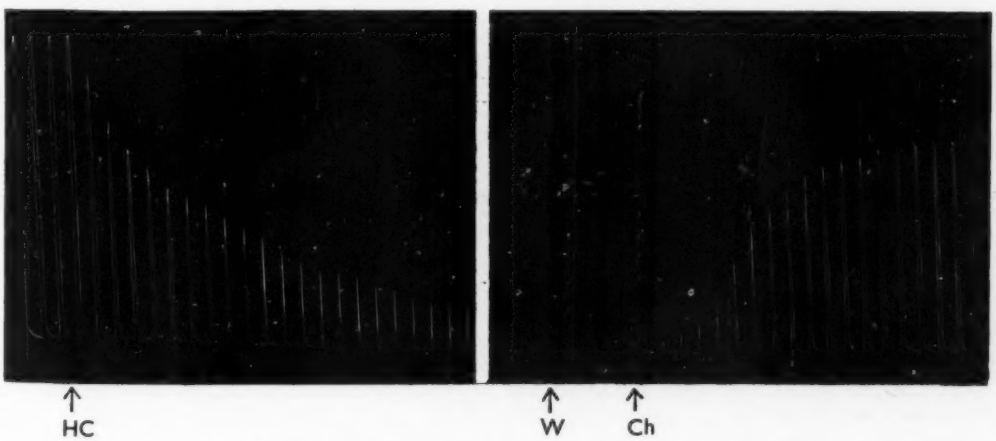


FIG. 1.—Guinea-pig vas deferens. Contraction in response to hypogastric nerve stimulation with 2 msec. pulses at 10/sec. for 10 sec./2 min. At (HC), 20 μg./ml. of hemicholinium was added to the bath, which produced a gradual decrease in response and complete failure 100 min. later in (*b*). The hemicholinium was washed out at (W), and after choline chloride (0.5 mg./ml.) was added at (Ch) the contractions were restored.

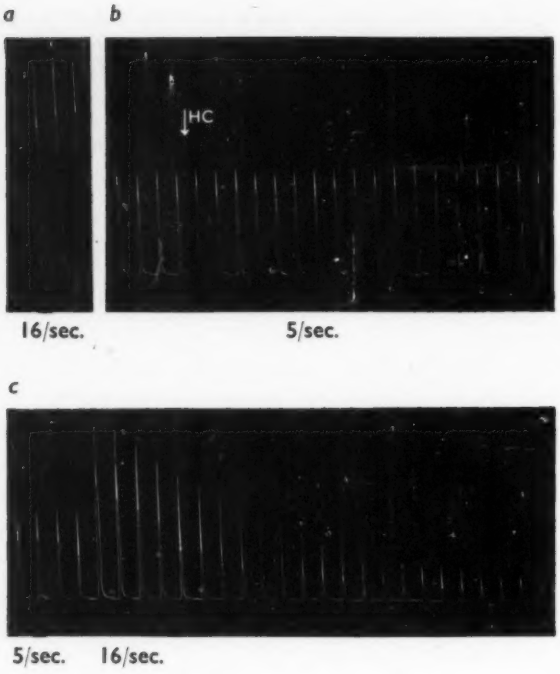


FIG. 2.—Guinea-pig vas deferens. Hypogastric nerve stimulated with 2 msec. pulses for 10 sec. periods every 2 min. In (*a*) the frequency of stimulation was 16/sec.; in (*b*) 5/sec. During stimulation at 5/sec., hemicholinium (30 μg./ml.) was added to the bath at (HC), and 100 min. later, in (*c*), the frequency was again altered to 16/sec. There was only a slight reduction of responses to stimulation at 5/sec., but at 16/sec. failure developed rapidly.

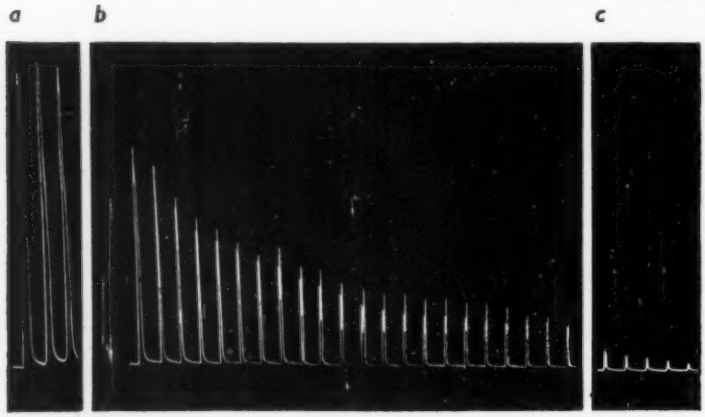


FIG. 3.—Guinea-pig vas deferens. In (*a*), hypogastric nerve stimulated at 16/sec. Between (*a*) and (*b*), hemicholinium (40 μg./ml.) was added to the bath and no stimuli were applied until 100 min. later. In (*b*), periods of stimulation at 16/sec. were recommenced. Failure was almost complete after 100 min. of stimulation (in *c*), that is, 200 min. after hemicholinium.

slight decrease in response. However, when the frequency of stimulation was increased to 16/sec. the responses rapidly decreased. Fig. 3 illustrates an experiment in which the nerve was not stimulated for 100 min. after adding hemicholinium (40 μg./ml.) to the bath. During this period hemicholinium produced a 30% decrease in the contraction in contrast to Fig. 1 in which the nerve was regularly stimulated and the response was completely blocked by hemicholinium after 100 min.

When hemicholinium had reduced, but not yet abolished contractions, we observed that the speed of the contraction was decreased, the contraction was not sustained, and the latent period of the response was increased. These effects are shown in Fig. 4 in which the responses were recorded at a faster kymograph speed.

After hemicholinium had produced failure of the response to nerve stimulation, choline chloride in concentrations of 0.1 to 1.0 mg./ml. in the bath gradually restored contractions (Fig. 1b). The observations made during the restoration of responses by choline were the reverse of those made during the development of failure by hemicholinium. The contractions gradually increased in size, were more sustained and the latent period decreased (Fig. 4).

The response of the vas deferens to noradrenaline was enhanced when the response to nerve stimulation had been abolished by hemicholinium (Fig. 5). The response of the vas deferens to nicotine was abolished as shown in Fig. 6b. However, the interpretation of this observation was not clear because choline restored the contractions in response to nerve stimulation to 61% of their original height, but only restored the response to nicotine to 15% of its original height (Fig. 6c). In other experiments it was found that hemicholinium immediately blocked the response to nicotine on the vas deferens, when the response to nerve stimulation was still unaffected. The response to acetylcholine was not affected by hemicholinium.

Atropine (3 μg./ml.) had no effect on the contractions of the vas deferens to hypogastric nerve stimulation before the addition of hemicholinium to the bath or after the restoration of responses by choline, although contractions produced by acetylcholine were blocked.

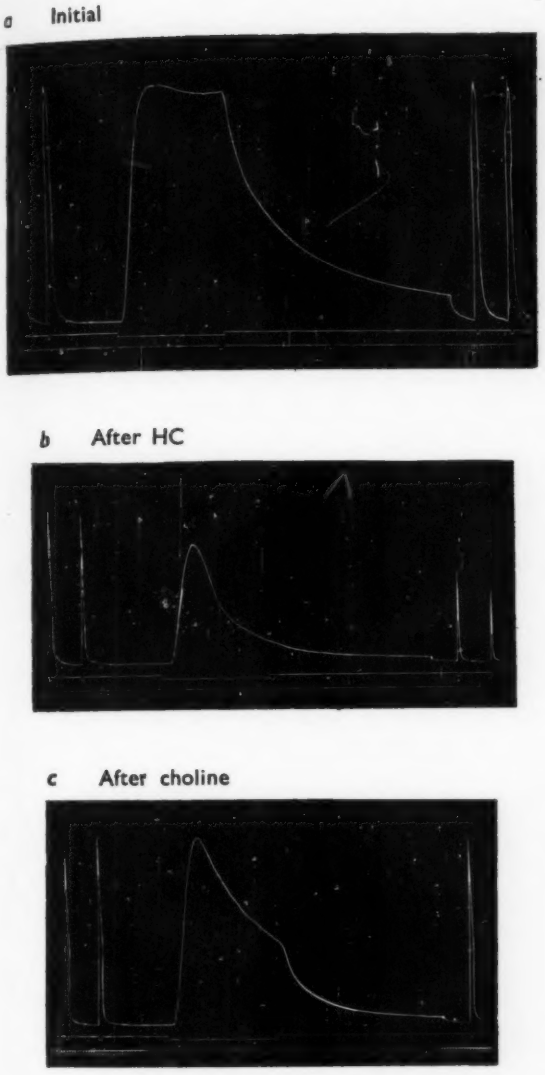


FIG. 4.—Guinea-pig vas deferens. In each panel, the upper trace is the record of contraction of vas deferens, the middle trace is a signal indicating the application of stimulus to the hypogastric nerve and the lower trace is a time signal showing 10 sec. intervals. The rate of kymograph movement was increased just before the 3rd response in each panel. Stimulation was with 2 msec. pulses at 16/sec., applied for 10 sec. in every 2 min. Control records are shown in (*a*). Between (*a*) and (*b*) 20 μg./ml. of hemicholinium was added to the bath, and (*b*) shows the record when the response had declined to about half of the control height. Between (*b*) and (*c*), 200 μg./ml. of choline chloride was added to the bath, and (*c*) shows the record when contractions were restored to about three-quarters of the control height.

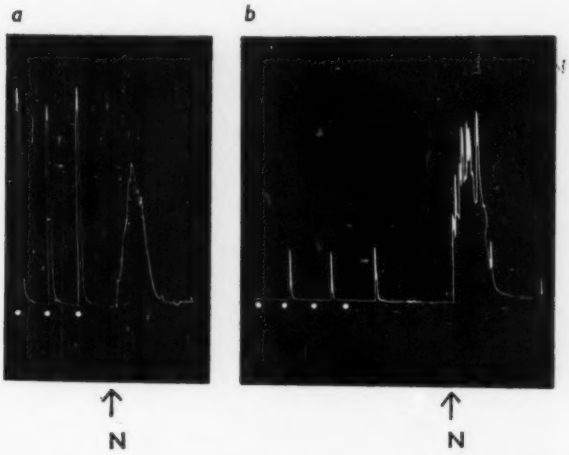


FIG. 5.—Guinea-pig vas deferens. The hypogastric nerve was stimulated at the white dots for 10 sec. at 10/sec. At (N) noradrenaline (10 μg./ml.) was added to the bath. Between (*a*) and (*b*), 20 μg./ml. of hemicholinium was added and stimulation was continued until failure was complete; the three small contractions in panel (*b*) were spontaneous.

Rabbit Uterus with Hypogastric Nerve.—The isolated rabbit uterus contracted regularly in response to hypogastric nerve stimulation with 2 msec. pulses at 25/sec. for 10 sec. in every 2 min. We confirmed Varagić's (1956) observation that the relaxation from the first contraction was slow, but after a few successive periods of stimulation the relaxation was more rapid.

The effect of hemicholinium (40 μg./ml.) is shown in Fig. 7. There was a gradually increasing irregularity of response to nerve stimulation; occasional responses reached the same height and were of even longer duration than observed initially, but other responses were small. The latent period of response after the start of nerve stimulation increased from 5 sec. to 15 sec. A second addition of hemicholinium to the bath to give a total concentration of 80 μg./ml. produced an increase of tone which appeared to be initiated by nerve stimulation and which then gradually declined. The responses to nerve stimulation were finally abolished, although some spontaneous rhythm of the uterus was present. The addition of choline chloride (800 μg./ml.) produced a contraction. After the choline was replaced by fresh McEwen solution the contractions of the uterus in response to nerve stimulation gradually recovered. At first the latent period was 15 sec. (i.e., contraction began 5 sec. after the end of the 10 sec. period of

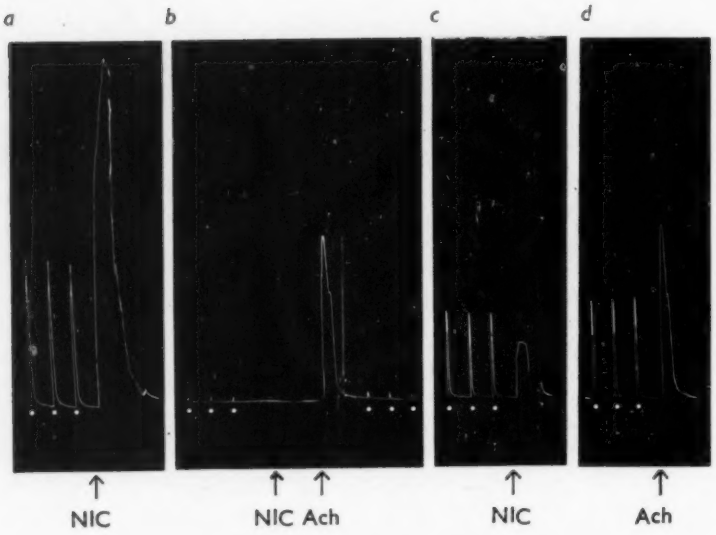


FIG. 6.—Guinea-pig vas deferens. Hypogastric nerve stimulation at white dots (12/sec. for 10 sec. periods). At (NIC), nicotine acid tartrate (20 μg./ml.), and at (Ach) acetylcholine chloride (20 μg./ml.) was added to the bath. Between (*a*) and (*b*) hemicholinium (20 μg./ml.) was added to the bath and stimulation was continued until failure developed. Between (*b*) and (*c*), choline chloride (0.4 mg./ml.) was added to the bath fluid and remained in (*c*) and (*d*).

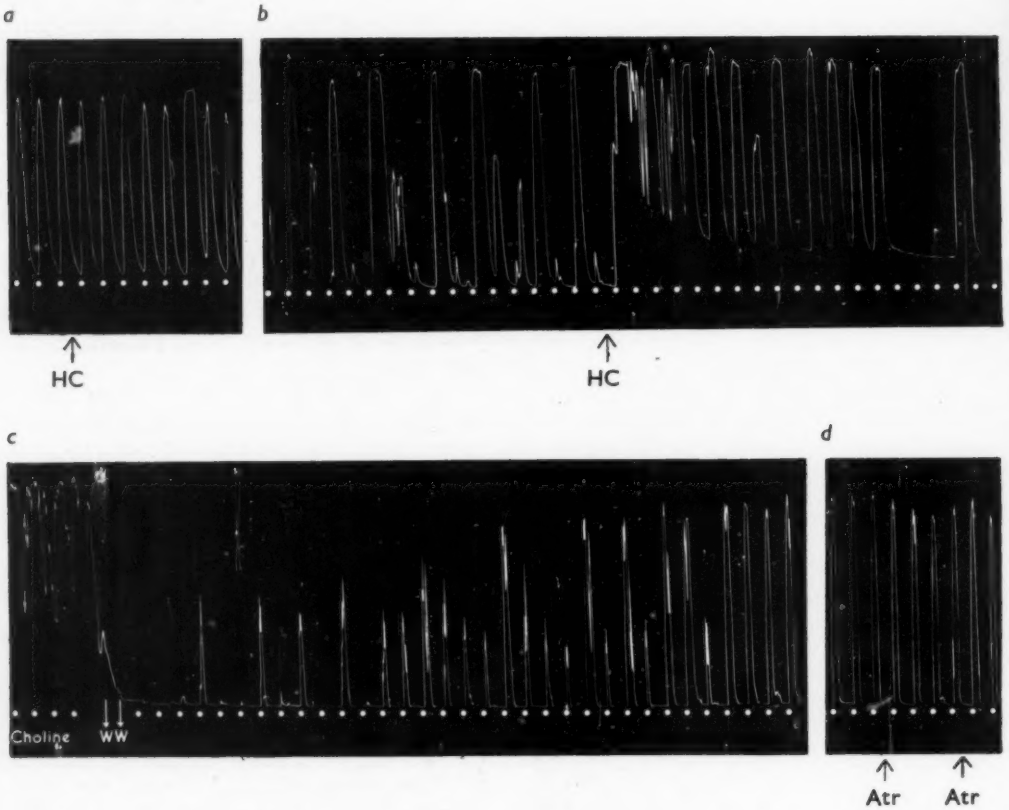


FIG. 7.—Isolated rabbit uterus. The hypogastric nerve was stimulated with 2 msec. pulses at 25/sec. for 10 sec. in every 2 min. as indicated by the white dots. In (*a*), hemicholinium (40 μg./ml.) was added to the bath at (HC). Between (*a*) and (*b*) 46 min. elapsed. In (*b*), a second addition of hemicholinium was made. Between (*b*) and (*c*), choline chloride (0.8 mg./ml.) was present in the bath, and it was washed out at W. Responses to nerve stimulation were gradually restored. In (*d*), atropine sulphate (Atr.) (0.5 and 1.0 μg./ml.) had no effect on the responses.

stimulation); when the contractions had been fully restored the latent period was 7 sec. The restored contractions to nerve stimulation were not affected by atropine (1 μg./ml.), a result which indicated that they were not produced by the direct action of acetylcholine from cholinergic nerve fibres on the uterine smooth muscle.

Rabbit Colon with Lumbar Sympathetic Nerves.—Constant relaxations of the colon were obtained in response to nerve stimulation at 25-50/sec. applied for 30 sec. in every 4.5 min.

The effect of hemicholinium (50 μg./ml.) in one experiment is illustrated in Fig. 8. The inhibition produced by sympathetic nerve stimulation was gradually reduced and almost abolished by the 20th period of stimulation after hemicholinium. In this experiment a slight restoration of the response was seen after washing out the hemi-

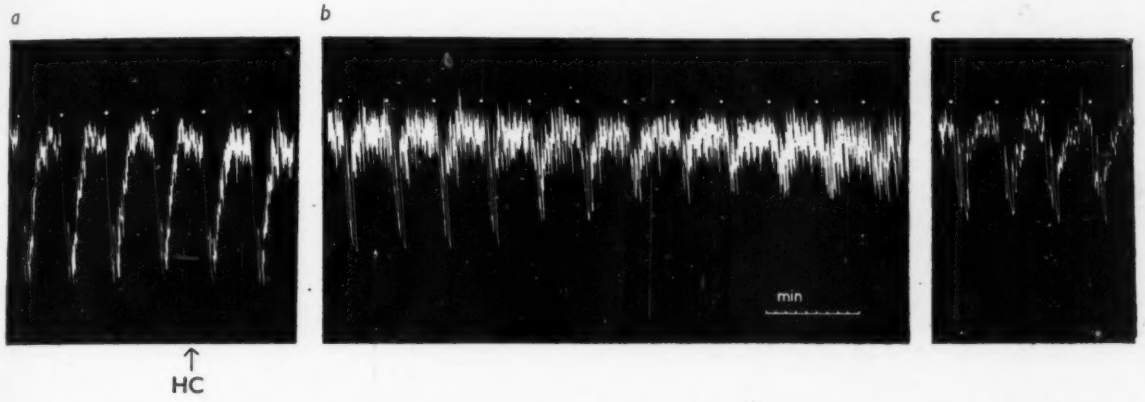


FIG. 8.—Rabbit distal colon in Krebs' bicarbonate solution. The sympathetic nerve supply to the colon was stimulated with 2 msec. pulses at 50/sec. for 30 sec. in every 4.5 min. at the white dots. In (*a*), hemicholinium (50 μg./ml.) was added at (HC). The interval between (*a*) and (*b*) was 45 min. Between (*b*) and (*c*), the bath was washed out and stimulation was stopped. In (*c*), 1 hr. later, relaxations were partly restored.

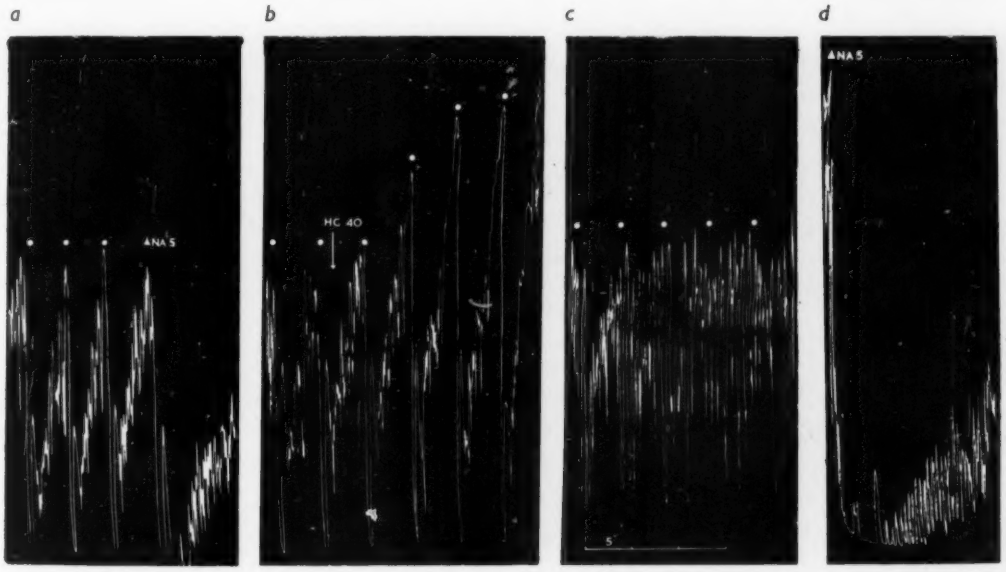


FIG. 9.—Rabbit colon in McEwen solution. The sympathetic nerves were stimulated with 2 msec. pulses at 25/sec. for 30 sec. in every 4.5 min. at the white dots. In (*a*) noradrenaline (5 μg./ml.) was injected at (NA) (white triangles). In (*b*), hemicholinium (40 μg./ml.) was added to the bath. In (*c*), 160 min. after hemicholinium, the relaxations in response to nerve stimulation were abolished, but in (*d*) noradrenaline still produced relaxation.

cholinium and resting from stimulation for one hour.

In another experiment hemicholinium produced an increase in tone of the colon (Fig. 9). At first the relaxations produced by nerve stimulation were greater than before hemicholinium, but subsequently the responses were abolished, although the tone remained enhanced and there was an increased amplitude of the spontaneous rhythm. The relaxation produced by noradrenaline was greater during a hemicholinium-induced failure of nerve stimulation than it had been initially.

Sympathetically Stimulated Perfused Rabbit Ear.—The vasoconstriction produced by postganglionic sympathetic stimulation was blocked by hemicholinium in each of 5 experiments. In 2 experiments a concentration of 20 μg./ml. of hemicholinium in the Locke solution perfusing the ear produced failure of the response to sympathetic stimulation in 1 hr. and in 6 hr., and in 3 experiments using hemicholinium in a concentration of 50 μg./ml. the response to sympathetic stimulation failed after 3 hr., 4 hr., and 6 hr. respectively.

After perfusing with hemicholinium the latency of response was increased. In one experiment in which stimulation was applied for 1 min. in every 5 min. the vasoconstriction reached its maximum after 30 sec. of stimulation, but 1 hr. after perfusing with hemicholinium (20 μg./ml.) the maximum response was reached after 45 sec. of stimulation.

In 2 experiments the response to sympathetic stimulation increased at first after perfusion with hemicholinium was begun (Fig. 10, panel *c*) and was subsequently abolished (panel *d*).

The response to sympathetic nerve stimulation could be partially restored by changing to fresh Locke solution and injecting choline into the arterial cannula at intervals (Fig. 11).

When the response to sympathetic stimulation had been abolished by hemicholinium the responses to noradrenaline (Fig. 11) and to nicotine (Fig. 10) were still present; in fact, the response to noradrenaline was somewhat larger than it had been and that to nicotine showed a considerable potentiation.

Isolated Cat Atria with Sympathetic Innervation.—Observations were made on the amplitude of contraction of the atria recorded with a light spring lever and on the rate of contractions. In Fig. 12 the stellate ganglion was stimulated with 2 msec. pulses at 20/sec. for 30 sec. in every 5 min. The increases in rate and amplitude of atrial beating were constant. The

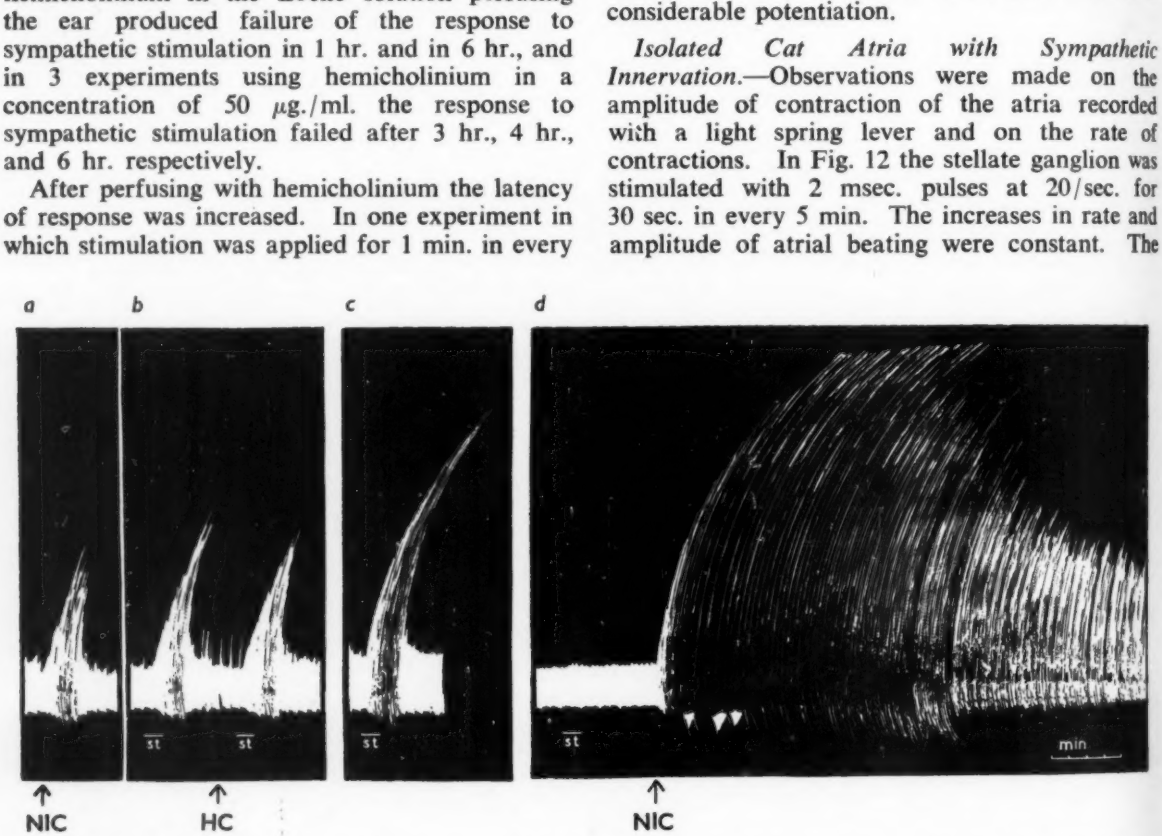


FIG. 10.—Outflow from perfused rabbit ear recorded by Gaddum's method. In (*a*) nicotine acid tartrate (1 mg.) was injected at (NIC). In (*b*) the superior cervical ganglion was stimulated at *st* with 2 msec. pulses at 50/sec. for 50 sec. Stimulation was repeated regularly at 5 min. intervals. In (*b*) hemicholinium (50 μg./ml.) was added to the perfusion fluid at (HC). Two hours later, in (*c*), the vasoconstriction produced by nerve stimulation was enhanced, but 6 hr. later, in (*d*), the response to nerve stimulation was abolished although the response to nicotine was greatly enhanced.

concentration of hemicholinium required to affect the responses to sympathetic stimulation was greater than in the preparations described above. After hemicholinium (500 μg./ml.) had been added to the bath the resting rate of atrial beating was increased and then gradually declined to its previous level. Initially, stimulation of the stellate ganglion produced an increase in rate of 50 to 58%. One hour after hemicholinium it was approximately 14%, and 3 hr. after hemicholinium there was no response. The latent period gradually increased during the development of failure of response. A slight recovery of response was seen when hemicholinium was washed from the bath and the preparation rested for 30 min.

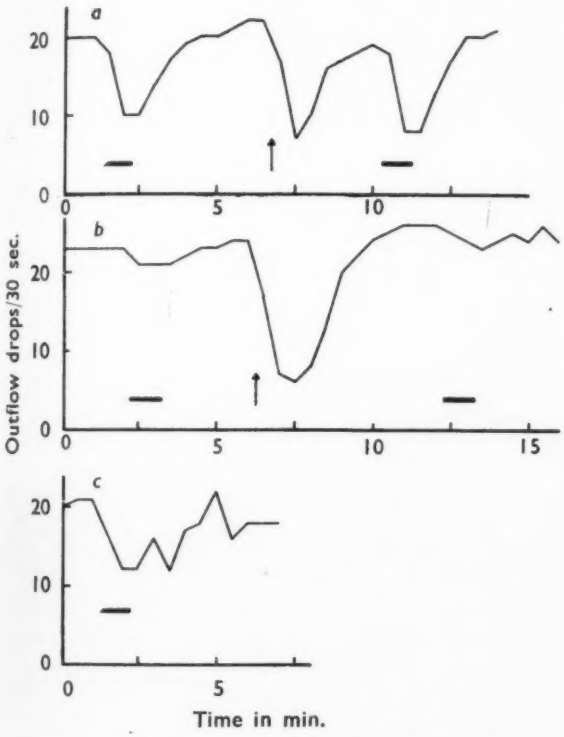


FIG. 11.—Outflow from perfused rabbit ear recorded in drops/30 sec. The horizontal lines indicate periods of stimulation of the superior cervical ganglion with 2 msec. pulses at 25/sec. for 50 sec. Periods of stimulation were repeated at 5 min. intervals throughout the experiment. At the arrows, noradrenaline (1 μg.) was injected into the arterial cannula. (*a*) shows the control observations; (*b*) is 3 hr. after perfusing with 50 μg./ml. of hemicholinium; the responses to nerve stimulation were abolished, but the response to noradrenaline was enhanced. Between (*b*) and (*c*) the ear was perfused with Locke solution without hemicholinium and four injections of 100 mg. of choline chloride were given. The stimulation applied in (*c*) was once more effective.

In another preparation (Fig. 13) the response to nerve stimulation was restored by adding choline to the bath. This slowed the rate of atrial contractions, but after the choline was washed out the responses to stellate ganglion stimulation were fully restored.

Piloerection in the Cat Tail.—Stimulation of the right lumbar sympathetic trunk with pulses at 20/sec. produced piloerection which reached its peak 2 sec. after the beginning of stimulation. The hairs were still maximally erected at the end of a 3 min. period of stimulation. Hemicholinium solutions were injected intradermally beneath alternate tufts of hair. In the experiment of Fig. 14, 0.05 ml. of each solution containing 500 μg./ml., 1 mg./ml., 2 mg./ml., and 5 mg./ml. of hemicholinium was injected beneath the tufts numbered 2, 4, 6, and 8 respectively. Tufts 1, 3, 5, 7, and 9 served as untreated controls.

Since the effect of hemicholinium in diminishing responses to nerve stimulation depended on the number of stimuli delivered, and since an increase in the latent period of the response has consistently been observed, the effect of hemicholinium was tested in the following manner. Photographs of the hair tufts were taken 3 sec. after the beginning of stimulation (Fig. 14, panel D); the tufts which had been injected with hemicholinium were slower to erect, although at the end of 10 sec. of stimulation the photograph shows that they were erected to the same extent as the untreated tufts (panel E). However, at the end of 3 min. of stimulation the tufts injected with hemicholinium had already partly relaxed, but the untreated tufts were still erected (panel F).

Rabbit Atria with Vagus Nerves.—The inhibition of atrial contractions in response to vagus nerve stimulation was reduced by hemicholinium (40 μg./ml.). In the experiment of Fig. 15 the effect of hemicholinium is shown on the response to vagal stimulation at 25/sec. for 1 min. periods in every 3 min. There was a clear reduction of responses at the 5th and 6th periods of stimulation and almost complete failure of response to the 10th and 11th periods of stimulation.

Responses to vagal stimulation were slightly restored after washing hemicholinium from the atria, and almost completely restored after treating the atria with choline chloride (0.4 mg./ml.).

Rabbit Isolated Vagina with Parasympathetic Innervation.—Regular contractions of the longi-

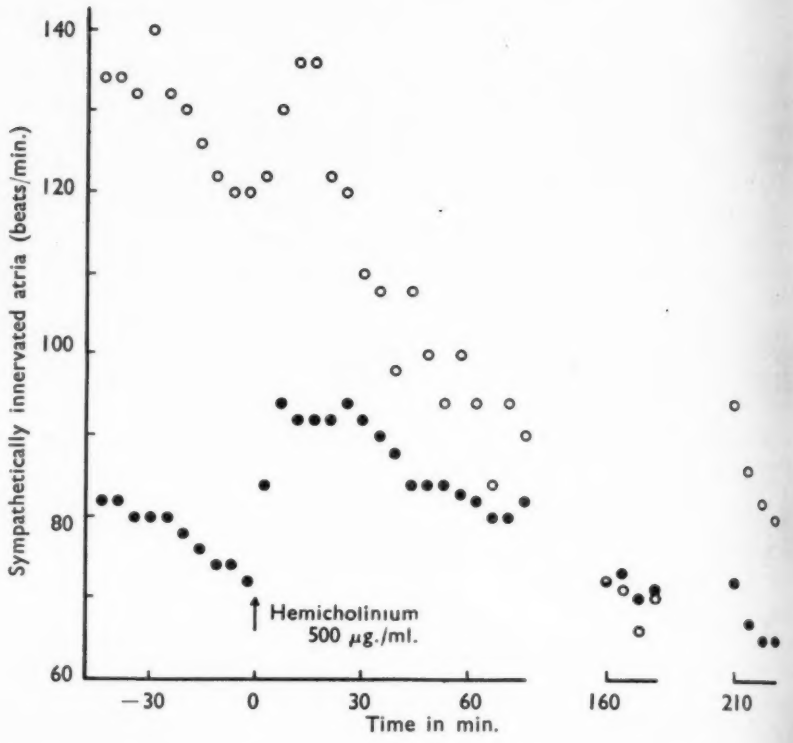
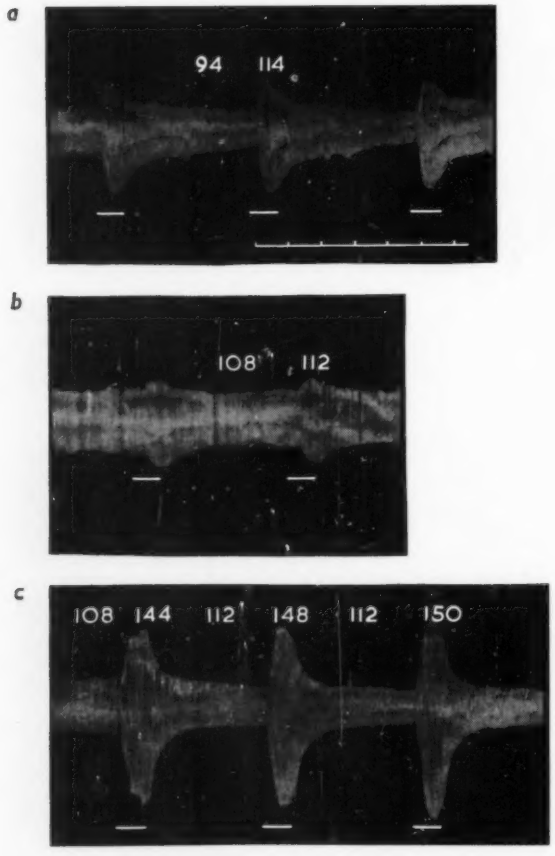


FIG. 12.—Rate of beating of isolated cat atria; ●, before sympathetic stimulation; O, during sympathetic stimulation. Sympathetic stimulation was by 2 msec. pulses at 20/sec. applied for 30 sec. at 5 min. intervals. After hemicholinium (500 μg./ml.) had been added to the bath the response to sympathetic stimulation decreased and was finally abolished. After 180 min. the bath was washed out and stimulation was stopped for 30 min., then recommenced.



tudinal muscle of the vagina were produced by stimulating the nerve for 10 sec. in every 2 min. Fig. 16 shows the effect of hemicholinium (24 μg./ml.) in inhibiting the contractions, and the restoration of response in the presence of choline chloride (400 μg./ml.). The restored responses were blocked by 0.4 μg./ml. of atropine, thus demonstrating that the contractions were due to the direct action of nervously released acetylcholine.

Guinea-pig Isolated Diaphragm with Phrenic Nerve.—Fig. 17 shows the effect of hemicholinium in diminishing the contractions of the diaphragm in response to phrenic nerve stimulation at the rate of 1/4.6 sec. After 40 min. in the presence of 200 μg./ml. of hemicholinium the diaphragm contracted to 20% of the height of the initial contraction. Choline chloride (1 mg./ml.) restored the contractions to their original height.

FIG. 13.—Isolated cat atria. The stellate ganglion was stimulated at 50/sec. for 50 sec. every 4.5 min. as indicated by the white lines. The numerals over the record refer to the rate in beats/min.; (*a*) shows initial observations. In (*b*), 4½ hr. after hemicholinium (500 μg./ml.), the response to stimulation was almost abolished. Between (*b*) and (*c*) the bath contained 1 mg./ml. of choline chloride, which was washed out before (*c*) was obtained. The time marker in (*a*) indicates 1 min. periods.

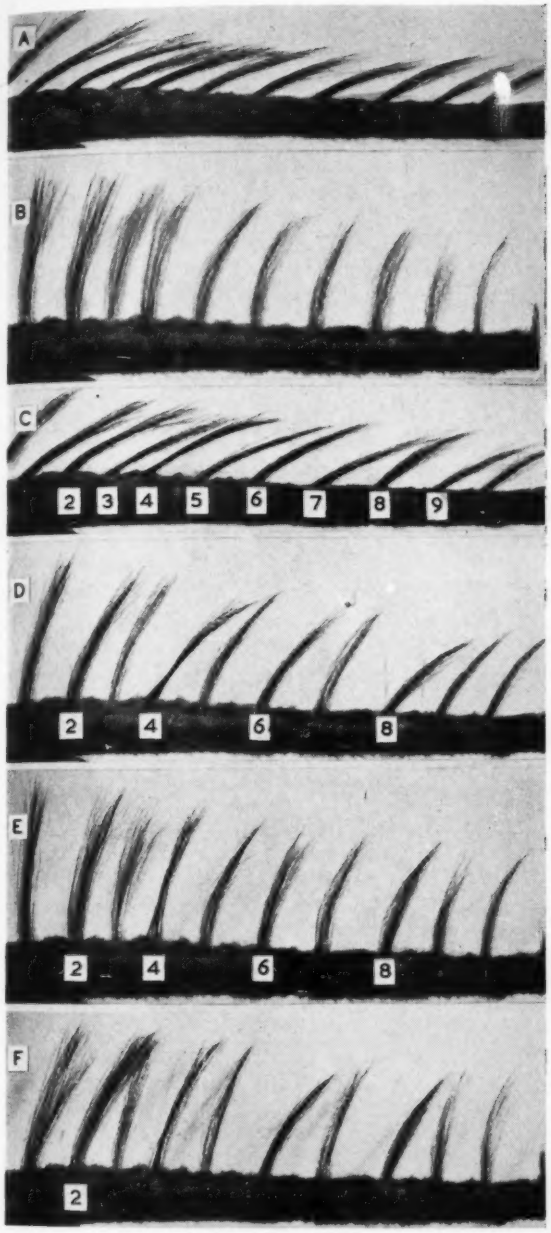


FIG. 14.—Photographs of tufts of hair on dorsal surface of cat's tail. (*A*) No stimulation. (*B*) Lumbar sympathetic trunk stimulated at 20/sec. for 10 sec. In (*C*) tufts 1, 3, 5, 7, and 9 were untreated; intradermal injections of hemicholinium were given beneath tufts 2 (0.5 mg./ml.), 4 (1 mg./ml.), 6 (2 mg./ml.), and 8 (5 mg./ml.). Between (*C*) and (*D*) several bursts of stimulation were applied. Then the lumbar sympathetic trunk was stimulated and (*D*) was taken after 3 sec. of stimulation, (*E*) after 10 sec. and (*F*) after 3 min. The tufts injected with hemicholinium were slower to erect and the erection did not persist during stimulation.

DISCUSSION

Hemicholinium was at first considered to act primarily on the central nervous system to produce depression of respiration (Long and Schueler, 1954; Schueler, 1955; Kasé and Borison, 1958). The findings of Longo (1958) that the phrenic nerve action potentials were not impaired when hemicholinium had abolished respiratory movements suggested that the inhibition of respiratory movement was peripheral, although the experiments of Kasé and Borison (1958) clearly indicate that there are also central effects. That hemicholinium blocks at the myoneural junction has been adequately shown by Long and his colleagues (Wilson and Long, 1959; Reitzel and Long, 1959b).

Reitzel and Long (1959b) reported that hemicholinium was more effective in blocking at the myoneural junction (sciatic nerve-gastrocnemius muscle) when the nerve was stimulated at 1/sec. than when it was stimulated at 1/10 sec. However, they pointed out that similar observations had been made for tubocurarine (Preston and Van Maanen, 1953; Gesler and Hoppe, 1956), although there were points of difference between the blockade produced by hemicholinium and that by tubocurarine, perhaps the principal one being that hemicholinium did not block the action of acetylcholine on the frog rectus muscle. Hemicholinium blocked transmission in the superior cervical ganglion and its blocking action depended on the frequency of preganglionic nerve stimulation (MacIntosh *et al.*, 1956). The ganglionic blocking drugs also have a greater blocking action against high frequencies of stimulation (Paton, 1951; Green, 1956). Thus there appears to be, in general, a relationship between the degree of block and the frequency of stimulation with a diverse range of blocking drugs, but the mechanisms involved may be very different.

Perry (1957) has distinguished seven phases in ganglionic transmission, and presumably all are susceptible to drug action leading to block. The difference in mechanism between a hemicholinium-induced failure of transmission and the blockade produced by the ganglionic blocking drug pentamethonium has been revealed by studies with the cat perfused superior cervical ganglion. MacIntosh *et al.* (1956) found that hemicholinium reduced the amount of acetylcholine released into the perfusion fluid on preganglionic nerve stimulation, which is in contrast to the finding of Paton and Zaimis (1951)

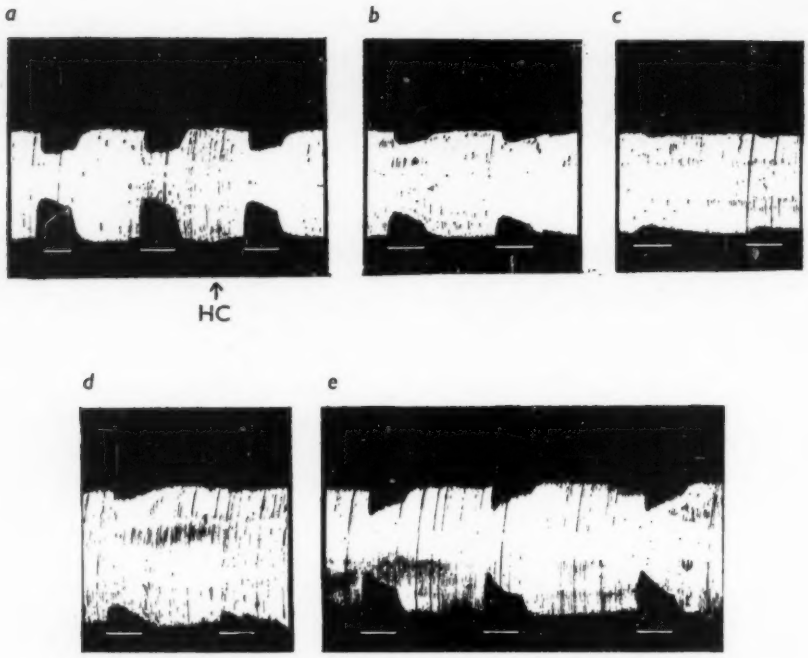


FIG. 15.—Isolated rabbit atria. The vagus nerves were stimulated at 20/sec. for 1 min. at 3 min. intervals as indicated by the horizontal lines. In (*a*) hemicholinium (40 μg./ml.) was added to the bath at (HC); (*b*) shows the 5th and 6th responses to vagal stimulation after hemicholinium and (*c*) shows the 10th and 11th. Between (*c*) and (*d*) the bath was washed out and stimulation was stopped for 2 hr. Between (*d*) and (*e*) choline chloride (0.4 mg./ml.) was added to the bath and then washed out.

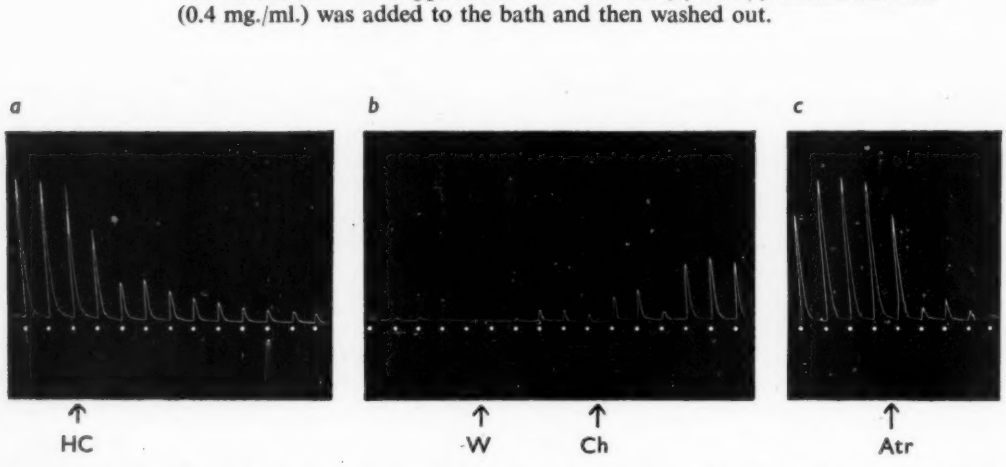


FIG. 16.—Isolated rabbit vagina. Parasympathetic nerve was stimulated at 60/sec. for 10 sec. in every 2 min. as indicated by white dots. Hemicholinium (24 μg./ml.) was added to the bath at (HC) in (*a*). In (*b*) the bath was washed out (W) 52 min. after hemicholinium, then choline chloride (0.4 mg./ml.) (Ch) was added, which restored responses. In (*c*) responses were blocked by 0.4 μg./ml. of atropine sulphate (Atr).

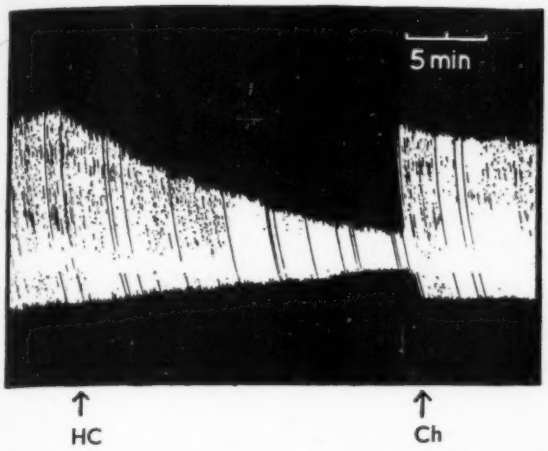


FIG. 17.—Contractions of guinea-pig diaphragm. The phrenic nerve was stimulated every 4.6 sec. At (HC) 200 μg./ml. of hemicholinium was added to the bath. At (Ch) the drum was stopped, the bath washed out and 1 mg./ml. of choline chloride was added. The drum was restarted 40 min. later.

that a blocking dose of pentamethonium did not interfere with the release of acetylcholine.

The blocking action of hemicholinium at the sympathetic nerve endings was only observed at high frequencies of stimulation. However, bretylium, whose blocking action is also exerted at sympathetic nerve endings, is also more effective at higher frequencies of stimulation (Boura and Green, 1959), although Nasmyth and Andrews (1959) found that the block produced by the related drug choline 2,6-xylyl ether did not depend on frequency. The effects of nicotine and of acetylcholine (in the presence of atropine) in constricting the vessels of the rabbit's ear are essentially similar (Kottegoda, 1953 ; Burn and Rand, 1958). One difference between the hemicholinium block at sympathetic nerve endings and the block produced by bretylium is that in the perfused rabbit ear nicotine produced vasoconstriction after hemicholinium had blocked sympathetic responses, whereas bretylium blocked both sympathetic responses and the vasoconstriction produced by acetylcholine (Huković, 1960a).

In the experiments of MacIntosh *et al.* (1956) with the cat perfused superior cervical ganglion, they found that hemicholinium decreased the rate of acetylcholine release into the perfusion fluid as the ganglion lost its ability to transmit impulses, and the store of acetylcholine in the ganglion was diminished. These effects of hemicholinium were reversed by the addition of choline to the perfusate. The synthesis of acetylcholine by mouse brain mince was inhibited by hemicholinium and the inhibition was reversed by choline (MacIntosh *et al.*, 1956, confirmed by Gardiner, 1957). However, the acetylcholine synthesizing ability of reconstituted acetone-dried powders of rat brain was less affected by hemicholinium. Gardiner (1957) concluded that the inhibiting action of hemicholinium was lessened when the degree of tissue disruption was increased. From these observations it was concluded that hemicholinium did not affect the enzyme choline acetylase, but that it acted on a system (carrier) which transported choline to the site of the acetylation (MacIntosh *et al.*, 1956 ; Gardiner, 1957 ; Birks and MacIntosh, 1957).

The reversal of hemicholinium block at the myoneural junction (sciatic nerve-gastrocnemius muscle) by choline has been extensively studied by Reitzel and Long (1959a). They found that choline itself was the only active substance in a series of choline analogues. Esters of choline which could be hydrolysed by cholinesterase were also effective, but their reversing action was abolished by the anticholinesterase TEPP, which suggested that the esters must first be split to yield choline. The reversing action of choline was enhanced when cholinesterase was blocked, which suggested that acetylcholine was formed from the choline. These observations support the conclusion of MacIntosh *et al.* (1956) that the transmission failure produced by hemicholinium was due to the impairment of acetylcholine synthesis, so that responses to nerve stimulation failed because acetylcholine became depleted, and that the reversal by choline was due to the restoration of acetylcholine synthesis.

Broadly speaking, one of two diametrically opposed conclusions arises from our findings that hemicholinium blocked responses to stimulation of sympathetic nerves. Firstly, it may be that hemicholinium exerts its nerve blocking activity through specific interference with acetylcholine synthesis. Alternatively, it may be that the sympathetic blocking action of hemicholinium and its reversal by choline are unrelated to acetylcholine, in which case arguments arising from the use of hemicholinium in the investigation of "cholinergic" nerves must be discounted. The similarity in the actions of hemicholinium in blocking sympathetic nerves and in blocking cholinergic nerves is striking: the effective concentrations of hemicholinium were approximately equal as far as isolated preparations are concerned, the block depended on the frequency of stimulation, and choline reversed the block.

We prefer a single theory to explain the action of hemicholinium on both "cholinergic" and "adrenergic" nerves rather than to suggest that it acts on "cholinergic" nerves by inhibiting the synthesis of acetylcholine and on "adrenergic" nerves in a different way.

The possibility that sympathetic effects may be mediated *via* acetylcholine has been discussed by Burn and Rand (1959, 1960b). Evidence has been obtained for cholinergic fibres in the sympathetic nerve supply to the vessels of the dog hind leg (Bülbring and Burn, 1935), to the cat nictitating membrane (Bacq and Fredericq, 1935; Burn and Rand, 1960b), to the rabbit ear (Burn and Rand, 1960b), to the rabbit ileum (Gillespie and Mackenna, 1959), to the rabbit atria (Huković, 1959), to the cat spleen (Burn and Rand, 1960b; Brandon and Rand, 1960), to the cat uterus (Burn and Rand, 1960b), to the rabbit uterus (Varagić, 1956), to the dog tongue and lips (von Euler and Gaddum, 1931), and to the guinea-pig vas deferens (Boyd, Chang, and Rand, 1960). In various organs acetylcholine (like nicotine) has sympathomimetic effects which are exerted at a site peripheral to the sympathetic ganglion cells, and it has been shown that these effects depend on the presence of noradrenaline stores in the organ (Burn, Leach, Rand, and Thompson, 1959). Therefore the possibility exists that the acetylcholine from sympathetic cholinergic fibres might be exerting a sympathomimetic effect by releasing noradrenaline.

The present findings that hemicholinium produces a block of responses to sympathetic nerve stimulation which is reversed by choline can be understood if acetylcholine were necessary at sympathetic nerve endings to release noradrenaline.

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ANALGESIC ANTIPYRETIC DRUGS AS ANTAGONISTS OF BRADYKININ

BY

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The antagonism between analgesic antipyretic drugs and bradykinin was examined quantitatively, using the bronchoconstrictor response of guinea-pigs *in vivo*. The dose of bradykinin required to overcome antagonism by calcium acetylsalicylate increased with the dose of acetylsalicylate given, the ratio being roughly constant. Fifty times the quantity of acetylsalicylate which just antagonized bradykinin did not modify bronchoconstriction due to small doses of histamine, 5-hydroxytryptamine, or acetylcholine. A method of measuring the potency of this anti-bradykinin action was developed. Acetylsalicylic acid, phenylbutazone, amidopyrine, and phenazone had a high potency; paracetamol, cinchophen, sodium salicylate, and acetanilide had a moderate potency; and phenacetin, salicylamide, and 4-hydroxyisophthalic acid had little or none. Cortisone, hydrocortisone, aldosterone, amodiaquine, and morphine were ineffective or their action was non-specific. In sensitized guinea-pigs, an injection of antigen caused bronchospasm. This response was greatly lessened by pretreatment with mepyramine, but was not affected by calcium acetylsalicylate, lysergic acid diethylamide, or atropine. Acetylsalicylic acid, phenylbutazone, and amidopyrine did not specifically antagonize the action of bradykinin on the capillaries of guinea-pig skin *in vivo*, on guinea-pig ileum *in vitro* or on rat duodenum *in vitro*.

Collier, Holgate, Schachter, and Shorley (1959, 1960) found that bradykinin causes bronchoconstriction in the guinea-pig, that small doses of acetylsalicylic acid, phenylbutazone and amidopyrine suppress this response without affecting those to histamine or 5-hydroxytryptamine, and that increasing the dose of bradykinin overcomes this suppression. Since the relationship between these analgesic and antipyretic drugs and bradykinin in its bronchoconstrictor action shows the main features of pharmacological antagonism, we thought it worth while to study this relationship quantitatively, to measure the potency of antagonism and to explore how effective were other antipyretic, analgesic, anti-inflammatory and anti-rheumatic drugs. Since bradykinin might take part in anaphylactic bronchospasm, we investigated whether pre-treatment with acetylsalicylate lessened the intensity of this spasm. We examined also whether the antagonists of bradykinin in its bronchoconstrictor action likewise antagonized some of its other actions.

METHODS

Materials.—Bradykinin was prepared by the action of crystalline trypsin on heated beef serum globulin and was purified chromatographically. The potencies of preparations, standardized against pure bradykinin obtained with trypsin (Elliott, Lewis, and Horton, 1960), ranged from 0.6 to 16 μg. pure bradykinin/mg. Substances tested in acute experiments as antagonists of bradykinin are given in Table III, which shows the salts used. Histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate and acetylcholine chloride were also employed as bronchoconstrictor agents and mepyramine maleate, lysergic acid diethylamide tartrate and atropine sulphate as their respective antagonists. Weights of salts are expressed as acid or base. Where possible, drugs were administered dissolved in 0.9% sodium chloride solution. Acetylsalicylic acid, salicylamide, 4-hydroxyisophthalic acid, cinchophen, acetanilide, phenacetin and amodiaquine were suspended in 5 to 10% gum acacia solution. Cortisone and cortisone acetate were suspended in 0.9% sodium chloride solution. Acetylsalicylic acid was given intravenously after mixing 10 parts by weight with three of calcium carbonate and one of citric acid and dissolving in 0.9% sodium chloride solution immediately before use. We refer to acetylsalicylic acid in this soluble form as calcium acetylsalicylate or more briefly as acetylsalicylate.

Guinea-pig Lungs in vivo.—To measure bronchoconstriction, we used the method of Konzett and Rössler (1940), with details as described by Collier *et al.* (1960). We estimated antagonism to bradykinin bronchoconstriction by giving intravenous

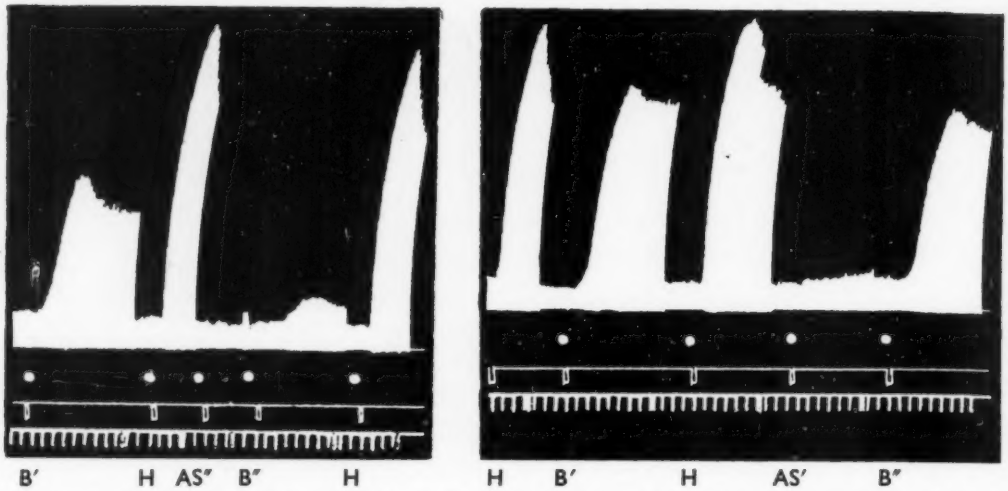

FIG. 1.—Resistance to inflation of guinea-pig lungs *in vivo*. Estimation of intravenous minimal effective dose of calcium acetylsalicylate. B′, 0.4 μg., and B″, 0.8 μg. of bradykinin; H, histamine, 2 μg.; AS′, 1 mg./kg., and AS″, 2 mg./kg. of calcium acetylsalicylate. All doses were given intravenously. Guinea-pig weights: left, 450 g.; right, 380 g. Time, 10 sec.

bradykinin and histamine before and after an intravenous, intraduodenal or oral dose of antagonist. Intravenous injections were spaced at 5 to 10 min. intervals. Bradykinin and histamine were injected 0.5 to 1 hr. after an intraduodenal or an oral dose of antagonist and subsequently. Because responses to repeated doses of bradykinin decline, the second dose of this substance was doubled. A dose of antagonist was considered effective if it reduced the second response to bradykinin to less than half the preceding one. An effective dose was regarded as specific if it did not depress the histamine response. Different doses of antagonist were given, each in a different guinea-pig, at a factor of 0.5 on the scale 512, 256, 128 . . . 4, 2, 1 and the minimal effective dose was taken as the lowest effective dose on this scale . This procedure is illustrated for acetylsalicylate in Fig. 1.

In some experiments, antagonists were administered daily for 3 to 4 days and then the bronchoconstrictor response to bradykinin compared with that of controls.

Three-week-old guinea-pigs were sensitized by injecting 10% egg albumen in saline, 1 ml. subcutaneously and 1 ml. intraperitoneally. About three weeks later, the effects of antagonists on anaphylactic bronchospasm were studied.

Guinea-pig Skin Capillaries in vivo.—We used the method of Miles and Miles (1952), applied to bradykinin by Holdstock, Mathias, and Schachter (1957). Guinea-pigs were depilated with Sleek (Elizabeth Arden) over a dorsal area lying between the pectoral and pelvic girdles and extending 4 cm. on either side of the mid-line Bradykinin and histamine were administered intradermally at 4 to 9 sites half to one hour after injecting intravenously 1.2 ml./kg. of 5% pontamine blue 6BX (Gurr) in saline. Half an hour after these control injections, antagonist was given intraperitoneally or subcutaneously and, half an hour later, bradykinin and histamine were again injected intradermally at other sites. All intradermal injections were randomized in relation to agent, site, dose, and time. Responses were measured as areas by tracing the zone of blueing 10 min. after intradermal injection on to mm. squared paper.

Guinea-pig Ileum in vitro.—Isotonic contractions were recorded from pieces of ileum, suspended in Tyrode solution in a 15 ml. bath at 32°.

Rat Duodenum in vitro.—Isotonic movements were recorded from pieces of isolated rat duodenum, suspended in de Jalon solution in a 15 ml. bath at 32°, according to the method of Gaddum and Horton (1959).

RESULTS

Resistance to Inflation of Guinea-pig Lungs

In 9 guinea-pigs, using three doses of acetylsalicylate, we determined approximately how much bradykinin was needed to restore the response 5 min. after acetylsalicylate to the same magnitude as that to 0.4 μg. of bradykinin before it. The results are expressed in Table I, which shows that the dose of bradykinin required to overcome antagonism by acetylsalicylate increased with the dose of acetylsalicylate given, the ratio at three doses being roughly constant. To save bradykinin, we did not give higher doses than 4 mg./kg. of acetylsalicylate.

Table II shows the percentage decrease in the response to bradykinin in 11 guinea-pigs, obtained by the procedure described for determining the minimal effective dose in the section on methods,

TABLE I

RESTORATION OF BRONCHOCONSTRICTOR RESPONSE OF GUINEA-PIGS TO BRADYKININ AFTER CALCIUM ACETYLSALICYLATE

A standard response was first obtained to 0.4 μg. of bradykinin. After administering acetylsalicylate at three doses, the doses of bradykinin were determined which evoked a response similar to the standard. All doses were given intravenously.

Dose of Acetylsalicylate (mg./kg.)	Dose of Bradykinin to Restore Response (μg.)	Dose-Ratio
1	1·6–3·2	4–8
2	6·4–12·8	16–32
4	12·8–25·6	32–64

using intravenous doses of 0.5, 1, or 2 mg./kg. of acetylsalicylate. From these results, if any of the observed responses to a particular dose were equally likely, the choice of the minimal effective dose would be 2 mg./kg. on two-thirds, 1 mg./kg. on one-quarter and 0.5 mg./kg. on one-twelfth of the occasions. From Tables I and II it will be seen that the minimal effective dose for intravenous acetylsalicylate (2 mg./kg.) corresponds to a dose-ratio (Gaddum, Hameed, Hathway, and Stephens, 1955) of 16 to 32. Cutting both vagi in the neck did not affect the potency of acetylsalicylate. This test was used to examine other potential antagonists of bradykinin. Table III gives each minimal effective dose which is based on determinations in at least three animals. Of the eight analgesic antipyretic drugs given intravenously, seven showed measurable anti-bradykinin activity, the descending order of potency being: calcium acetylsalicylate, phenylbutazone, amidopyrine and phenazone, paracetamol, cinchophen and sodium salicylate. Of the eleven drugs given orally, only five were active. We therefore gave drugs by the intraduodenal route which Corne and Edge (1958) had found better than the oral for pempidine in anaesthetized cats. Table III shows that sodium

TABLE II

REDUCTION OF BRONCHOCONSTRICTOR RESPONSE OF GUINEA-PIGS TO BRADYKININ BY SINGLE DOSES OF CALCIUM ACETYLSALICYLATE

In 11 guinea-pigs a reference response was first obtained to 0.4 μg. of bradykinin. After administering acetylsalicylate, a response to 0.8 μg. bradykinin was obtained. The % reduction was measured by the extent to which the second response to bradykinin was reduced in comparison with the first. All doses were given intravenously.

% Reduction of Bradykinin Response at Doses of Acetylsalicylate		
0·5 mg./kg.	1·0 mg./kg.	2·0 mg./kg.
−12	14	56
−9	37	61
26	50	82
68		100

TABLE III

POTENCIES OF VARIOUS AGENTS IN SUPPRESSING BRONCHOCONSTRICTION DUE TO BRADYKININ IN GUINEA-PIGS

The minimal effective dose (MED) is the least dose of an antagonist that reduces the response to an intravenous dose of bradykinin, which is twice the preceding dose, to less than half the preceding response, without reducing that to histamine. * Administered as calcium salt; § administered as sodium salt; —, not tested; I.P., intraperitoneal; I.M., intramuscular.

Agent	MED (mg. Acid or Base/kg.)			
	Intravenous	Oral	Intraduodenal	Other Routes
Acetylsalicylic acid	2*	32	64	—
Sodium salicylate	64	>512	256	—
Salicylamide ..	—	>256	>512	—
4-Hydroxyisophthalic acid	>64§	—	>512	I.P >512
Cinchophen ..	32§	>512	256	—
Sodium phenylbutazone ..	4	16	16	—
Amidopyrine ..	8	16	16	—
Phenazone ..	8	64	128	—
Acetanilide ..	—	>512	256	—
Phenacetin ..	—	>512	512	—
Paracetamol ..	16	512	128	—
Amodiaquine phosphate ..	—	>512	>512	—
Cortisone ..	—	—	—	I.M. >25
Hydrocortisone sodium succinate	Non-specific at 100	—	—	—
D,L-Aldosterone	>2	—	—	—
Morphine sulphate ..	>32	—	—	—
1-(1-Phenylcyclohexyl)piperidine hydrochloride ..	>16	—	—	—

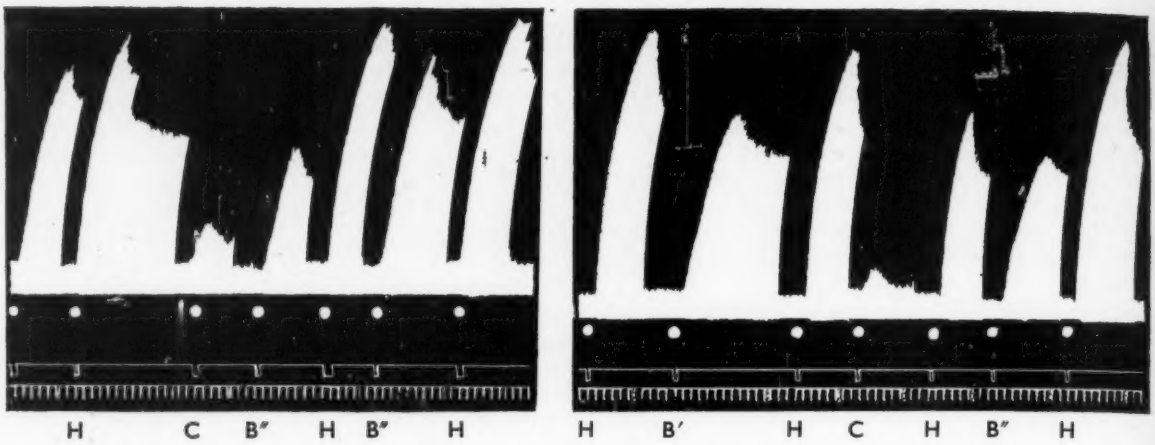

FIG. 2.—Resistance to inflation of guinea-pig lungs *in vivo*. Depression of responses to bradykinin and histamine by a large dose of hydrocortisone. B′, 0.4 μg., and B″, 0.8 μg. of bradykinin; H, histamine, 1.0 μg.; C, hydrocortisone sodium succinate, 100 mg./kg. All doses were given intravenously. Guinea-pig weights: left, 515 g.; right, 650 g. Time, 10 sec.

salicylate, cinchophen, acetanilide and phenacetin were active intraduodenally though not by mouth.

Amodiaquine in single large oral or intraduodenal doses was ineffective. Repeated administration of this drug did not lower the response of guinea-pig bronchioles to bradykinin. Two guinea-pigs each received by mouth 50 mg./kg. amodiaquine daily on 4 successive days. One hour after the last treatment, the bronchiolar responses of each animal to bradykinin were tested and found to be normal. In a similar subacute experiment with chloroquine phosphate in 3 guinea-pigs, bronchiolar responses also remained normal.

Since a single intramuscular dose of cortisone (25 mg./kg.) failed to reduce the bradykinin response (Table III), 3 guinea-pigs were given daily on 3 successive days 25 mg. of cortisone acetate subcutaneously. One hour after the third dose, the bronchiolar responses of each animal were within normal limits. Another 3 guinea-pigs received 100 mg./kg. of hydrocortisone sodium succinate intravenously. Five minutes after treatment, bronchiolar responses both to histamine and bradykinin were reduced (Fig. 2), but only for about 15 min.

We investigated how far acetylsalicylate and amidopyrine, given intravenously, antagonized

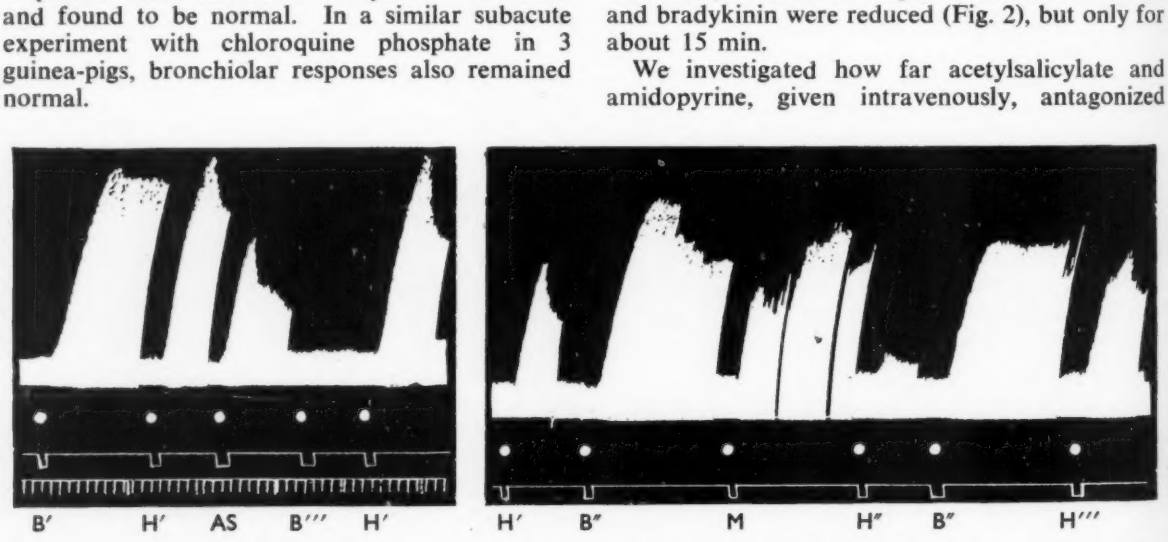

FIG. 3.—Resistance to inflation of guinea-pig lungs *in vivo*. Failure of calcium acetylsalicylate to suppress response to histamine and of mepyramine to suppress response to bradykinin. B′, 0.4 μg., B″, 0.8 μg., and B‴, 1.6 μg. of bradykinin. H′, 1 μg., H″, 1 mg., and H‴, 2 mg. of histamine. AS, calcium acetylsalicylate, 100 mg./kg. M, mepyramine maleate. 10 mg./kg. All doses were given intravenously. Guinea-pig weights: left, 460 g.; right, 500 g. Time, 10 sec.

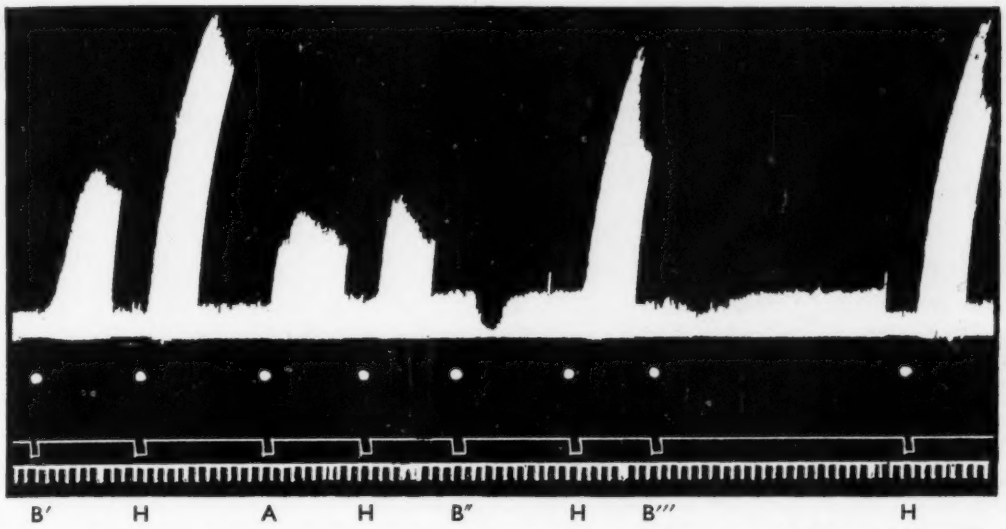

FIG. 4.—Resistance to inflation of guinea-pig lungs *in vivo*. Degree of specificity of amidopyrine towards bradykinin. B′, 0.4 μg., B″, 3.2 μg., and B‴, 6.4 μg. of bradykinin. H, histamine, 2 μg. A, amidopyrine, 100 mg./kg. All doses were given intravenously. Weight of guinea-pig, 580 g. Time, 10 sec.

bradykinin specifically. While 2 mg./kg. of acetylsalicylate effectively reduced the response to bradykinin, 100 mg./kg. did not alter that to 1 μg. histamine (Fig. 3). In other experiments, 100 mg./kg. of acetylsalicylate did not reduce the responses to 2 μg. of 5-hydroxytryptamine or 2 μg. of acetylcholine. Fig. 3 also shows that as much as 10 mg./kg. mepyramine intravenously failed to affect the response to 0.8 μg. of bradykinin. Lysergic acid diethylamide (1 mg./kg.) and atropine (1 mg./kg.), both given intravenously, also failed to affect the response to bradykinin. At 8 mg./kg., amidopyrine effectively reduced the bradykinin response (Table III), while 50 mg./kg. lowered by one-quarter and 100 mg./kg. by two-thirds the response to 2 μg. of histamine (Fig. 4). Figs. 3 and 4 show that high doses of acetylsalicylate, amidopyrine, and mepyramine themselves caused bronchoconstriction. After giving amidopyrine (Fig. 4), acetylsalicylate or phenylbutazone, an apparent bronchodilator action of bradykinin was also sometimes revealed.

Effect of Drugs on Response to Antigen.—De Schaepdryver (1950), using the preparation of Konzett and Rössler (1940), found that antigen

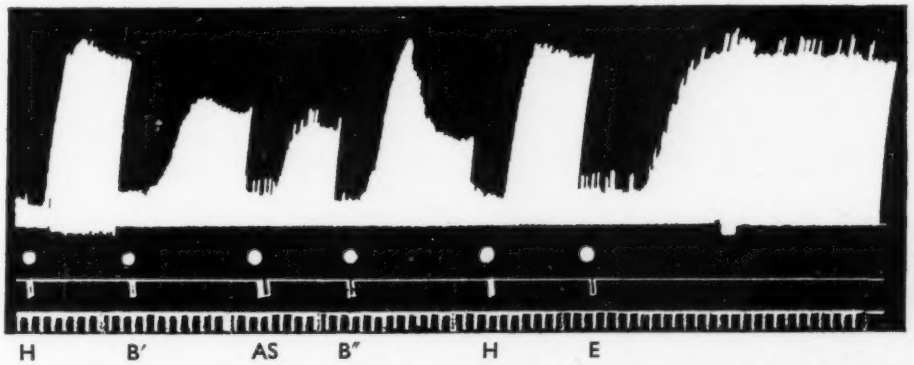

FIG. 5.—Resistance to inflation of guinea-pig lungs *in vivo* in an animal sensitized to egg albumen. Failure of a large dose of calcium acetylsalicylate to suppress bronchoconstriction due to antigen. B′, 0.4 μg., and B″, 12.8 μg. of bradykinin. H, histamine, 1 μg. AS, calcium acetylsalicylate, 100 mg./kg. E, egg albumen, 1 mg. All doses were given intravenously. Weight of guinea-pig, 340 g. Time, 10 sec.

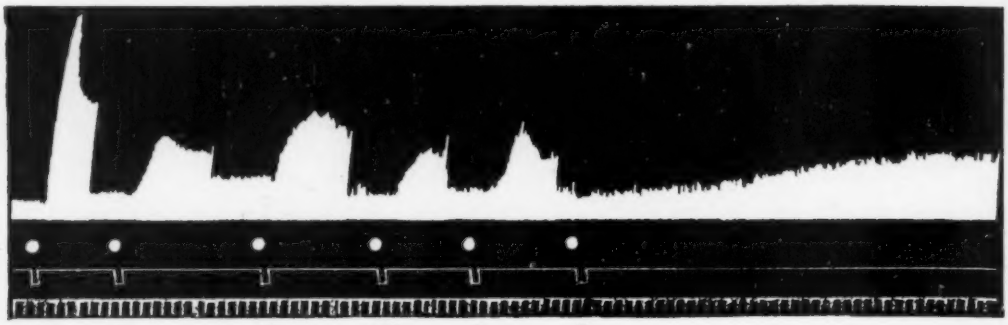

FIG. 6.—Resistance to inflation of guinea-pig lungs *in vivo* in an animal sensitized to egg albumen. Partial suppression by mepyramine of response to antigen. B, bradykinin, 0.4 μg. H′, 1 μg., and H″, 0.5 mg. of histamine. M, mepyramine, 5 mg./kg. E, egg albumen, 1 mg. All doses were given intravenously. Weight of guinea-pig, 420 g. Time, 10 sec.

injected intravenously produced a powerful bronchospasm in guinea-pigs sensitized to egg albumen. We have confirmed this. In 34 sensitized guinea-pigs, prepared for recording resistance of the lungs to inflation, antagonists of known bronchoconstrictor agents were given intravenously before egg albumen. Although acetylsalicylate (100 mg./kg.) markedly reduced the response to a large dose of bradykinin, it did not affect that to antigen (Fig. 5). Lysergic acid diethylamide (1 mg./kg.) and atropine (1 mg./kg.) were likewise ineffective against anaphylactic bronchospasm, although they readily suppressed respectively the responses to 5-hydroxytryptamine and acetylcholine. Mepyramine (0.5 to 5 mg./kg.) greatly lessened the response to antigen, but a small residual bronchoconstriction still remained (Fig. 6).

Skin Blueing in Guinea-pigs

In control experiments, we found that the curve relating area of skin blueing to intradermal dose of bradykinin had a flat slope, while that of

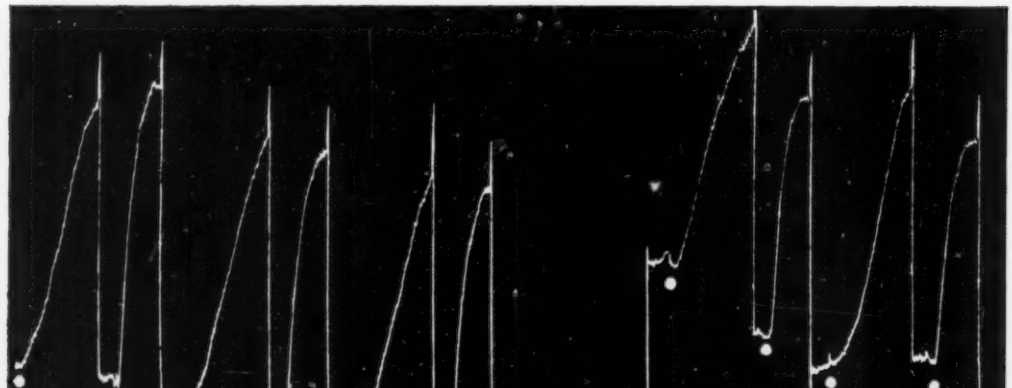

FIG. 7.—Isolated guinea-pig ileum in 15 ml. bath. Suppression by amidopyrine of responses to bradykinin and histamine. B, bradykinin, 0.24 μg. H, histamine, 0.2 μg. A, amidopyrine, 100 μg./ml. W, amidopyrine washed out.

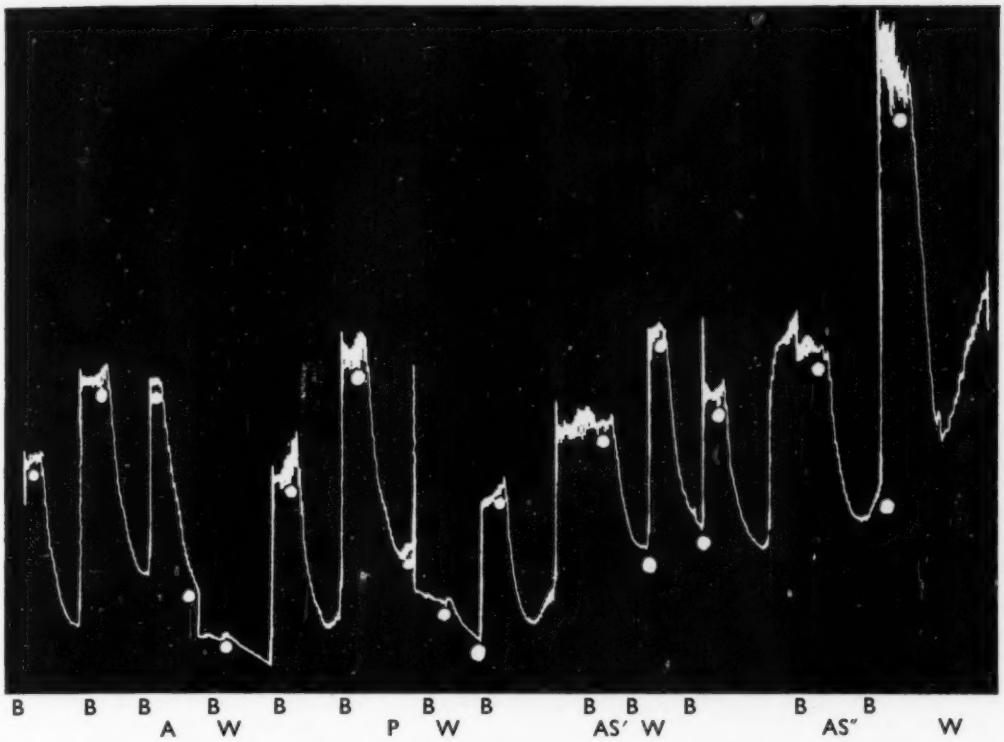

FIG. 8.—Isolated rat duodenum in 15 ml. bath. Failure of calcium acetylsalicylate to suppress response to bradykinin. B, bradykinin, 0.04 μg. A, amidopyrine, 50 μg./ml. P, phenylbutazone, 200 μg./ml. AS′, 200 μg./ml., and AS″, 800 μg./ml. of calcium acetylsalicylate. W, wash.

histamine had a steeper slope. This agrees with the observations of Bhoola, Calle, and Schachter (1960). Acetylsalicylic acid (150 and 200 mg./kg.) did not lessen the response of the capillaries to bradykinin or histamine, while mepyramine (0.5 mg./kg.) abolished the response to histamine, but left that to bradykinin unaffected (Table IV). Phenylbutazone (100 and 200 mg./kg.) and amidopyrine (75 and 150 mg./kg.) lessened but did not abolish responses to bradykinin and histamine, the effects on the histamine response appearing somewhat greater.

Intestinal Preparations in vitro

Phenylbutazone (200–400 μg./ml.), amidopyrine (20–100 μg./ml.), phenazone (200–400 μg./ml.) and cinchophen (200 μg./ml.) reduced the response of the isolated guinea-pig ileum to bradykinin. However, the same concentrations of these drugs also lessened the response to histamine, as will be seen for amidopyrine in Fig. 7. Acetylsalicylate (200 μg./ml.) failed to affect responses of the ileum to either agent.

TABLE IV

FAILURE OF ACETYLSALICYLIC ACID TO REDUCE AREAS OF SKIN BLUEING OF GUINEA-PIGS BY BRADYKININ

Animals were treated intraperitoneally with 200 mg./kg. of acetylsalicylic acid or with 0.5 mg./kg. of mepyramine after measurement of control areas of blueing.

Agent	Intradermal Dose (μg.)	Mean Area of Blueing (sq. mm.)			
		Before Acetylsalicylic Acid	After Acetylsalicylic Acid	Before Mepyramine	After Mepyramine
Histamine	0·025	22·5	27·5	13·5	0
	1·0	40	40	60·5	0
Bradykinin	0·002	27	22	31·0	31·5
	0·2	32	37·5	35·5	26·0

Amidopyrine (50 μg./ml.) and phenylbutazone (200 μg./ml.) relaxed the isolated rat duodenum, but did not prevent a further small relaxation after bradykinin (Fig. 8). Acetylsalicylate (200 and 800 μg./ml.) increased the tone of the rat duodenum, but did not suppress the relaxation due to bradykinin (Fig. 8). These drugs affected the response to vasopressin in the same way as that to bradykinin.

Discussion

We have used the term dose-ratio (Gaddum *et al.*, 1955) to describe the ratio of doses of agonist giving equal effects before and after antagonist.

We have studied the effects of acetylsalicylic acid, phenylbutazone and amidopyrine on four different biological responses to bradykinin. In one of these—bronchoconstriction in the guinea-pig *in vivo* — these drugs showed towards bradykinin several features of pharmacological antagonism. (1) Non-toxic doses of antagonist suppressed the response to the agonist; (2) larger doses of agonist restored the response; (3) when the dose of agonist was increased, larger doses of antagonist were effective; and (4) effective doses of antagonist did not reduce responses to other bronchoconstrictor agents. In another response—contraction of the guinea-pig ileum *in vitro*—phenylbutazone and amidopyrine decreased the effect of bradykinin, but affected equally the response to histamine. In two biological systems—the skin capillaries of blued guinea-pigs *in vivo* and rat duodenum *in vitro*—large doses of these drugs failed to suppress the response to bradykinin. In three of these preparations which responded to histamine, mepyramine specifically antagonized this substance.

The fact that some drugs specifically antagonized bradykinin in its bronchoconstrictor but not in its other actions has parallels in the antagonism of acetylcholine, histamine, 5-hydroxytryptamine and adrenaline. For example, different types of antagonists block acetylcholine at different sites of action, such as at the skeletal neuromuscular junction, at synapses in autonomic ganglia and at the nerve effector cell junctions in intestinal muscle. Such differences are generally ascribed to the existence of several types of receptor for acetylcholine. The same explanation may well apply to our observations on bradykinin.

The apparent bronchodilator action of bradykinin, which is unmasked in the presence of antagonists of its bronchoconstrictor action (Fig. 4), might be attributed to an impurity in the bradykinin preparations used. Alternatively and more probably it may be due to the presence in the bronchioles of a second type of receptor for bradykinin. This supposition would be consistent with the recent observation of Waaler (1960) that bradykinin does not constrict but sometimes slightly dilates the bronchioles of the isolated lung of the dog. This second type of receptor might perhaps correspond to that involved in the effects of bradykinin other than those blocked by antagonists of its bronchoconstrictor action.

It seems relevant to ask how far the degree of the antagonism we have observed is related to the other pharmacological actions of the antagonists. Winder, Wax, Burr, Been, and Rosiere (1958), Adams (1960), and Winder (personal communication) determined the degree to which orally administered drugs suppressed the skin erythema caused by ultra-violet irradiation of guinea-pigs. They showed that phenylbutazone, acetylsalicylic acid, amidopyrine and phenazone exhibit high to moderate anti-inflammatory potency in this test (Table V). Sodium salicylate, cinchophen and amodiaquine had low but detectable activity, while salicylamide, 4-hydroxyisophthalic acid, acetanilide, phenacetin, paracetamol, cortisone, cortisone acetate and hydrocortisone had little or no action. These results show some parallelism with our find-

Table V

POTENCIES OF VARIOUS AGENTS IN SUPPRESSING SKIN ERYTHEMA OF GUINEA-PIGS DUE TO ULTRA-VIOLET IRRADIATION

Approximate "effective doses" in mg./kg. orally are derived from Winder *et al.* (1958), Adams (1960), and Winder (personal communication). Values in column headed Winder *et al.* are total doses. An asterisk indicates subcutaneous route; — indicates not tested.

Drug	Winder *et al.*	Adams
Acetylsalicylic acid ..	100 to 200	80
Sodium salicylate	200	120
Salicylamide	>400	>320
4-Hydroxyisophthalic acid	>400	>320
Cinchophen	200	—
Phenylbutazone	18	10
Amidopyrine	130	80
Phenazone	100	—
Acetanilide	>400	>240
Phenacetin	400	>240
Paracetamol	>400	>240
Amodiaquine	200	—
Chloroquine	>400	—
Cortisone	>25	—
,, acetate	>75	—
Hydrocortisone acetate ..	>75*	—

ings in antagonizing bradykinin. Marked activity in both tests is restricted to drugs of the analgesic-antipyretic type, the most potent, apart from acetylsalicylic acid, being pyrazoline or pyrazolidine derivatives which have several chemical features in common. For the eleven agents of this type that we examined, a Spearman rank correlation coefficient between the potencies obtained by Winder *et al.* (1958) (Table V) and those in our test after intraduodenal administration had a value of 0.761, showing a probability of <0.01 that the two sets of potencies are independent. Since, however, intradermal bradykinin did not produce erythema in guinea-pigs and the skin blueing response did not show comparable antagonism by the drugs used, the interpretation of this correlation needs further study.

Winder *et al.* (1958) have called attention to a parallelism between the performance of analgesic-antipyretic drugs in suppressing ultra-violet erythema in guinea-pigs and the clinical value of these drugs in rheumatism. Since our results correlate well with those Winder *et al.* (1958), it is not surprising that parallelism can also be discerned between suppression of bradykinin bronchoconstriction in guinea-pigs and anti-rheumatic activity of these drugs in man. Paracetamol, which is not active in the test of Winder *et al.* (1958) and which is not generally used in rheumatism, provides an exception. With this drug, however, recovery of the bradykinin response was evident within one hour of its intraduodenal administration. This brevity of action may account for the ineffectiveness of the drug in the ultra-violet light erythema test.

In animals, 4-hydroxyisophthalic acid has somewhat stronger analgesic and antipyretic properties than acetylsalicylic acid (Collier and Chesher, 1956); but Adams (1960) has called attention to the difference between these drugs in their abilities to suppress ultra-violet light erythema. The contrast in the potencies of these two drugs as antagonists of bradykinin supports the conclusion of Adams (1960) as does also the failure of 4-hydroxyisophthalic acid to benefit rheumatoid arthritis (Hajnal, Sharp, and Popert, 1959).

Outside the group of analgesic antipyretic drugs discussed above, there was no correlation between anti-rheumatic and anti-bradykinin action. The corticosteroids failed to antagonize bradykinin and so did chloroquine and amodiaquine, which are reported to be useful in rheumatoid arthritis (Haydu, 1953; Freedman, 1956; Pomeroy, Warren, Mills, and Clark, 1959; Bepler, Baier, McCracken, Rentschler, Rogers, and Lansbury, 1959; Kersley and Palin, 1959).

Chen and Weston (1960) have reported that 1-(1-phenylcyclohexyl) piperidine causes analgesia in the monkey; but this compound and morphine were inactive in our test. There was, therefore, no correlation between analgesic action as such and antagonism to bradykinin.

The failure of acetylsalicylate to lessen anaphylactic bronchospasm suggests that bradykinin does not play an important part in this form of bronchoconstriction. This finding is consistent with that of Herxheimer (1955) that intramuscular doses of phenylbutazone and of salicylic acid do not protect sensitized guinea-pigs against antigen given by aerosol. In our experiments mepyramine greatly reduced anaphylactic bronchospasm, as might have been expected from the observation of Armitage, Herxheimer, and Rosa (1952) that this drug protected sensitized guinea-pigs against antigen administered by aerosol.

Note Added in Proof

We examined how far acetylsalicylate antagonized a specimen of the bradykinin-like synthetic nonapeptide H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH (Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer; *Experientia*, 1960, **16**, 326), which Dr. E. D. Nicolaides and Dr. H. A. De Wald prepared. Assays of this sample against purified specimens of bradykinin (supplied by Dr. D. F. Elliott, Dr. G. P. Lewis, and Dr. E. W. Horton) gave comparable results on guinea-pig ileum, rabbit blood pressure and rat uterus, showing the sample contained approximately 70–90% active nonapeptide. Acetylsalicylate (2 mg./kg. intravenously) suppressed the bronchoconstrictor effect of the nonapeptide to a comparable extent to that of an equiactive dose of bradykinin. Larger doses of the nonapeptide overcame the suppression as did bradykinin in the same preparation. After acetylsalicylate both nonapeptide and nearly pure bradykinin showed some bronchodilator action.

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MEMBRANE POTENTIAL CHANGES ASSOCIATED WITH TACHYPHYLAXIS AND POTENTIATION OF THE RESPONSE TO STIMULATING DRUGS IN SMOOTH MUSCLE

BY

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(RECEIVED OCTOBER 3, 1960)

Conditions which affect the response of smooth muscle to repeated application of stimulating drugs have been investigated. In guinea-pig taenia coli, tension changes were recorded simultaneously with membrane potential changes using the sucrose gap technique. Acetylcholine, histamine, and 5-hydroxytryptamine caused depolarization and, after removal of the drug, hyperpolarization which was followed by a sequence of damped oscillations of the membrane potential. The average rate of depolarization decreased in the order acetylcholine > histamine > 5-hydroxytryptamine. The readiness with which tachyphylaxis occurred increased in the order acetylcholine < histamine < 5-hydroxytryptamine. When a dose of a stimulating drug was repeated, the response obtained depended on the phase of the oscillatory potential changes during which it was applied. In general the effect was depressed during a phase of polarization and enhanced during a phase of depolarization. The degree of tachyphylaxis—or potentiation—depended not only on the direction in which the membrane potential changed at the moment of drug application, but also on the relation between the rate at which this potential change took place and the rate of depolarization caused by the drug. The results observed are consistent with the hypothesis that the fluctuating excitability and polarization of the smooth muscle membrane is brought about by periodical changes in the rate of active ion transport and other stabilizing processes in the cell membrane which depend on the rate of metabolic energy supply. The muscle was sensitized to acetylcholine and histamine by previous treatment with, or in the presence of, 5-hydroxytryptamine.

Barsoum and Gaddum (1935) pointed out that the desensitization of smooth muscle preparations by repeated exposure to a stimulating drug, known as tachyphylaxis, represents a difficulty as well as a help in biological assay. A difficulty, because a previously effective dose may not produce a comparable contraction on repetition; a help, because the action of another drug may not be reduced to the same extent and it can thus be detected and identified if the two drugs are present in a mixture. Cantoni and Eastman (1946) investigated the possibility that, in the course of repeated drug application, the muscle contraction was reduced by exhaustion due to a previous maximal contraction. They recorded the response to a weak concentration of acetylcholine at regular intervals and found that it was depressed if the muscle was at one time exposed to a 100 times stronger concentration which caused a maximal contraction. This temporary depression was also observed after maximal contractions in response to large concentrations of histamine, barium chloride, pilocarpine and mecholyl. If, however, the maximal contraction was obtained by a large dose of potassium chloride, the acetylcholine contractions were not diminished, instead they were increased. Rand (1957), who repeated these observations, advanced the hypothesis that the responsiveness of the smooth muscle depended on the ratio of intracellular to extracellular potassium. While this ratio would be increased when normal solution was admitted after prolonged exposure to excess potassium, it would be reduced when the muscle had lost potassium after treatment with acetylcholine. Hughes, McDowall and Soliman (1956) found that changes in the sodium chloride concentration affected the response to repeated doses of histamine; tachyphylaxis was abolished by low sodium, but intensified by high sodium in the bathing solution.

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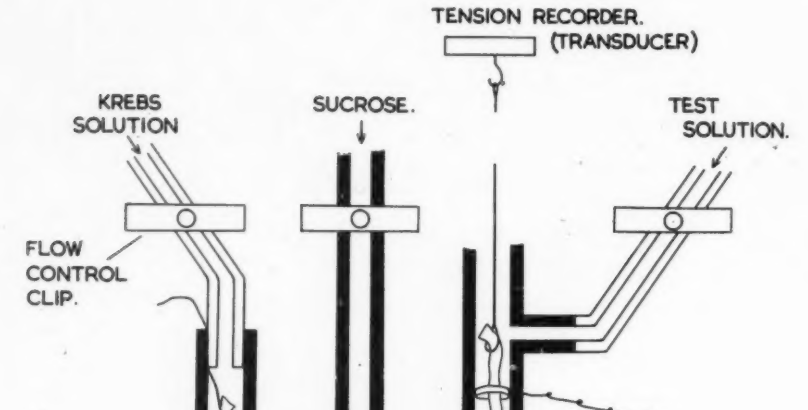

FIG. 1.—Diagram of apparatus used for extracellular recording by sucrose-gap method, modified to include stimulating electrodes and means for recording the tension.

The authors interpreted their observations by assuming that tachyphylaxis was due to a difficulty in sodium extrusion.

The present work was undertaken to investigate further the conditions leading to a change of the response to a stimulating drug by applying acetylcholine, histamine and 5-hydroxytryptamine for short periods in rapid succession and recording the mechanical responses as well as the changes in membrane potential and electrical activity.

A short account of the results was given at the meeting of the British Pharmacological Society on January 11, 1959.

METHODS

Ganglion-free intestinal smooth muscle taken from the taenia coli of the guinea-pig was used in all experiments and bathed by a modified Krebs' solution (Bülbring, 1953).

Extracellular recording from smooth muscle by the "sucrose gap" method (Stämpfli, 1954) has been described previously (Burnstock, 1958a; Burnstock and Straub, 1958). A modification of the chamber so that the muscle could be stimulated electrically has also been described (Bülbring, Burnstock, and Holman, 1958). In the present experiments a further alteration of the apparatus was made so that tension changes could be recorded electrically simultaneously with those of the membrane potential. The test side of the chamber was constructed so that solutions flowed through a side tube while the end of the muscle was attached by a vertical thread to a mechano-electronic transducer valve (RCA 5734) mounted in the manner described by Bülbring (1955). Care was taken to ensure that the flow of solutions was constant. The modified chamber is shown in Fig. 1.

RESULTS

Changes During Brief Exposure and Following the Removal of Stimulating Drugs

The changes of membrane potential, spike discharge and tension produced by short application of acetylcholine or histamine in intestinal smooth muscle are known to be qualitatively similar (Bülbring, 1955). The course of events is represented diagrammatically in Fig. 2*a*. Initially, after administration of the drug, there is a period of depolarization during which there is no spike discharge (or no increase in rate if spikes are already present) and no change of tension. This period, from A to B, will be called "lag." At a critical level (marked by an asterisk) spikes appear and consequently the tension rises. The period taken to reach the maximum tension, from A to C, will be called the "maximum response time." The maximum depolarization often coincides but sometimes occurs a few seconds before or later. After the maximum values are reached during the presence of the drug there is usually a slight repolarization and loss of tension. When the drug is washed out (at D)

the spike discharge is slowed and it soon stops (marked by asterisk), at which point the tension falls to the original level or slightly below. The membrane potential recovers more slowly and usually it also reaches a higher value than that recorded initially. The period taken from the time of washing out the drug to the maximum polarization of the membrane, from D to E, will be called the "maximum polarization time." The membrane potential then fluctuates in the pattern of a damped oscillation (see Fig. 4, to be discussed later) before it returns to the original value. Thus full recovery is not reached within the time course shown in the diagram of Fig. 2*a*. A typical record of the events following the administration of a drug (histamine 1×10^{-7}) for 45 sec. and the first stage of recovery after its removal is shown in Fig. 2*b*.

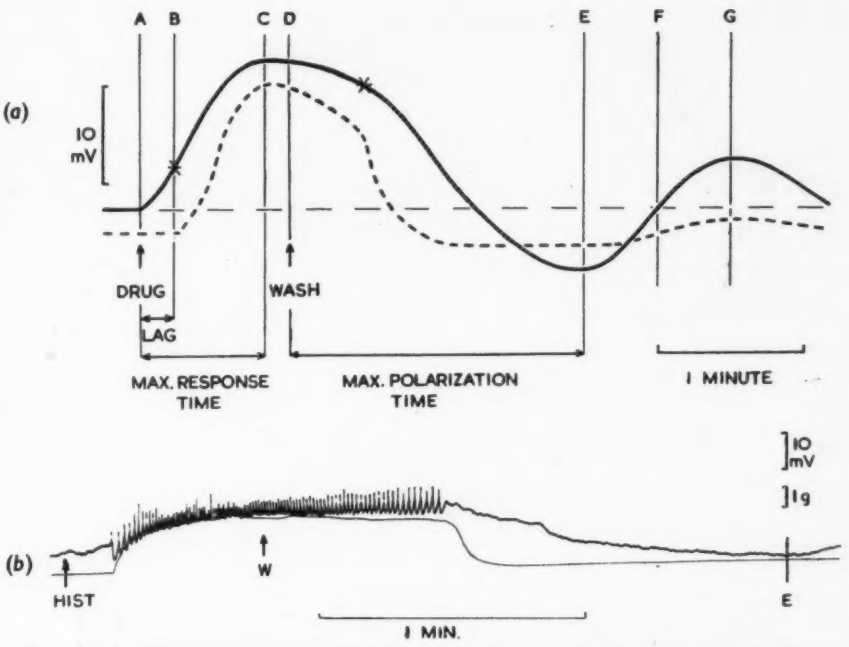

FIG. 2.—(*a*) Diagram representing the course of events following administration of a stimulating drug. Continuous line, membrane potential; broken line, tension. For description see text. (*b*) Records of electrical changes (upper trace) and tension changes (lower trace) produced by histamine 10^{-7}.

Comparison of the Responses to Acetylcholine, Histamine and 5-Hydroxytryptamine

The taenia coli was found to be most sensitive to 5-hydroxytryptamine and least sensitive to acetylcholine, for which 10^{-7} was sometimes the threshold concentration. Histamine 10^{-7} produced the most constant change in tension and this was, in different experiments, about equal to that caused by acetylcholine 10^{-7} to 10^{-6} and 5-hydroxytryptamine 2×10^{-9} to 5×10^{-8}. Equiactive concentrations of the three drugs, judged by the tension response, were applied to compare the periods defined above. The "lag" was shortest for acetylcholine and longest for 5-hydroxytryptamine. This was due to the difference in the rate of depolarization which decreased in the order acetylcholine > histamine > 5-hydroxytryptamine, as shown in Fig. 3. The period taken to reach the maximum tension was also shortest for acetylcholine and longest for 5-hydroxytryptamine. In Fig. 3, for example, the maximum response to acetylcholine occurred after 24 sec., to histamine after 61 sec., and to 5-hydroxytryptamine after 81 sec. (not shown in the record).

Whereas both the lag and the maximum response time showed consistent differences between the three drugs tested, the maximum polarization time after removal of the drug did not. In general, this period was prolonged when the dose and the duration of application of any given drug was greater. These results are summarized in Table I.

Comparison of the Responses to Different Concentrations of the Same Drug

With increasing concentrations of acetylcholine and histamine the lag became progressively shorter and the maximum response was reached earlier. However, as may be seen from Table I, the lag of the response to the greatest concentration of histamine (10^{-5}) was only about that of the weakest concentration of acetylcholine (10^{-7}). Furthermore, with a hundredfold increase in concentration, the average rate of depolarization caused by acetylcholine was increased four times, whereas the rate of depolarization caused by histamine was only increased by 50%. Thus the peak of the response to acetylcholine was reached between 35.9 and 27.7 sec., while that to histamine occurred much later, between 61.4 and 49.6 sec. Also for 5-hydroxytryptamine the lag became

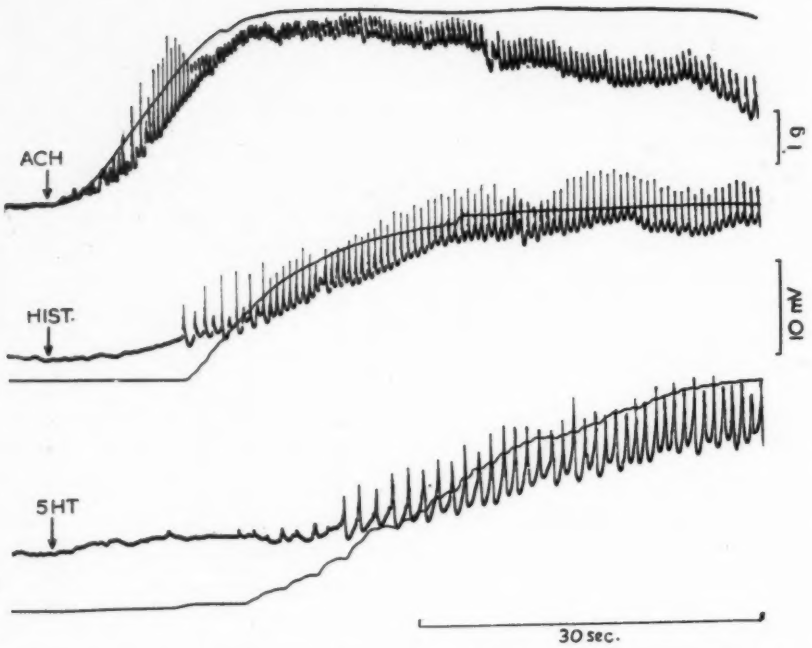

FIG. 3.—Records as in Fig. 2*b*. Comparison of the lag, of the rate of depolarization and of the maximum response time of equiactive concentrations of acetylcholine (ACH: 10^{-6}), histamine (10^{-7}) and 5-hydroxytryptamine (5HT: 5×10^{-8}). For description see text.

shorter with increasing concentrations, but the time taken to reach the maximum response became longer as the doses increased. With excessive concentrations of 5-hydroxytryptamine (such as 10^{-6}) the depolarization was usually preceded by a period of hyperpolarization. This was also observed when lower concentrations of 5-hydroxytryptamine were applied repeatedly for periods longer than 90 sec. A similar initial hyperpolarization was seen when high concentrations of histamine (namely 10^{-5}) were used.

We also compared the mean rates of repolarization following application of different concentrations of the same drug (Table I). After acetylcholine the rate of repolarization was about half that of depolarization. The

TABLE I

MAXIMUM RESPONSE TIME, AVERAGE RATES OF DEPOLARIZATION AND OF REPOLARIZATION IN THE TAENIA COLI OF THE GUINEA-PIG

The numerals in brackets are standard errors of the mean.

Drug and Conc.		Lag (sec.)	Maximum Response Time (sec.)	Average Rate of Depolarization (mV/sec.)	Average Rate of Repolarization (mV/sec.)	No. of Observations
Acetylcholine	10^{-7}	6·2 (±0·95)	35·9 (±2·08)	0·174 (±0·027)	0·097 (±0·021)	20
	5×10^{-7}	5·3 (±0·77)	35·7 (±1·05)	0·313 (±0·032)	0·106 (±0·016)	9
	10^{-6}	4·2 (±0·68)	31·9 (±2·50)	0·531 (±0·069)	0·323 (±0·143)	6
	10^{-5}	2·3 (±1·13)	27·7 (±3·25)	0·716 (±0·103)	0·173 (±0·012)	5
Histamine	10^{-7}	19·0 (±2·16)	61·4 (±2·23)	0·219 (±0·019)	0·173 (±0·026)	18
	10^{-6}	11·7 (±1·41)	60·6 (±5·74)	0·212 (±0·014)	0·214 (±0·044)	4
	10^{-5}	5·8 (±0·79)	49·6 (±7·09)	0·327 (±0·057)	0·158 (±0·028)	5
5-Hydroxy-tryptamine	2×10^{-9}	22·8 (±2·2)	62·0 (±5·94)	0·156 (±0·012)	0·161 (±0·023)	7
	1×10^{-8}	22·9 (±5·38)	72·0 (±7·42)	0·151 (±0·022)	0·100 (±0·011)	7
	5×10^{-8}	15·8 (±2·46)	72·5 (±9·49)	0·299 (±0·071)	0·242 (±0·050)	13
	1×10^{-7}	14·0 (±5·08)	86·0 (±10·0)	0·124 (±0·037)	0·084 (±0·020)	3
	5×10^{-7}	8·3 (±2·50)	140·0 (±20·0)	0·079 (±0·017)	0·372 (±0·175)	3
	1×10^{-6}	115·0 (±13·2)	280·0 (±20·0)	0·029 (±0·005)	0·043 (±0·009)	3

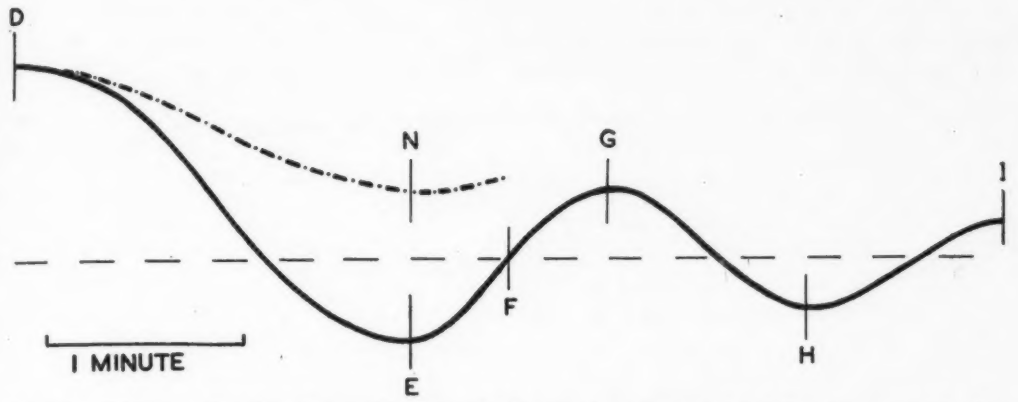

FIG. 4.—Diagram showing the oscillations of the membrane potential following removal of a stimulating drug at *D*. For description see text.

greater the depolarization caused by increasing acetylcholine concentrations the faster was the repolarization. After histamine and 5-hydroxytryptamine the rate of repolarization was very similar to the rate of depolarization. After exposure to excessive concentrations of 5-hydroxytryptamine (5×10^{-7} to 10^{-6}) repolarization was faster than depolarization.

Tachyphylaxis

The pattern of changes during recovery as already mentioned consisted of a phase of repolarization usually beyond the initial membane potential, and a subsequent period in which the membrane potential fluctuated in the manner of a damped oscillation. This period is shown diagrammatically in Fig. 4 (continuous line), where it has been divided into sections so that the time of application of the second dose could be specified in relation to the membrane oscillation. (Occasionally the membrane remained depolarized for a longer time; this condition is represented by the broken line DN.)

The time taken for recovery and the pattern of changes following exposure of the tissue to a drug depended both on the concentration used and on the duration of its application. Thus in experiments designed to determine the conditions causing tachyphylaxis these two factors were varied as well as the interval between doses.

Impairment of the response to a second dose of a drug could be detected by two criteria, namely the magnitude of the response and the delay before the response occurred. Thus tachyphylaxis was either evident from a decreased tension change, or it could be detected by a long lag, or it was shown by both criteria.

All three drugs, when applied during the first phase of polarization (period DE), showed tachyphylaxis which was evident, in nearly every experiment, from the greatly increased lag. With 5-hydroxytryptamine the tension was always correspondingly reduced; but in a number of experiments with histamine, and especially with repeated application of low concentrations of acetylcholine, the tension response was as great as before despite the increased lag. This was due to the fact that the "maximum response time" for acetylcholine was short. Consequently, though the onset of the response was delayed, the peak was reached before the drug was removed.

During the period EF, when the membrane potential was still hyperpolarized in relation to the initial level but had passed the peak of polarization, there was usually no reduced mechanical response to acetylcholine and rarely to histamine, but the lag was still prolonged. With 5-hydroxytryptamine tachyphylaxis was evident both from the longer lag and from the reduction of the mechanical response.

After this phase the tension change produced by acetylcholine and histamine was no longer impaired. Indeed, during FG or HI, when the membrane was depolarizing, a potentiation of the acetylcholine and histamine response often occurred. In contrast, the response to 5-hydroxytryptamine was even then diminished.

During the second polarizing phase of the recovery oscillations, GH, a longer lag was often observed once more. But while the mechanical responses to acetylcholine and histamine were not reduced, the response to 5-hydroxytryptamine was usually diminished even in the later stages of the oscillations, suggesting that some more permanent change had taken place. Typical examples of the progressive change of the responses to repeated doses are shown in Table II.

TABLE II

RESPONSES TO DRUGS ADMINISTERED IN CERTAIN OF THE PHASES OF OSCILLATION SHOWN IN FIG. 2

Expt. No.	Conc.	Duration of Exposure (sec.)	Duration of Interval (sec.)	Stage of Oscillation	Lag (sec.)	Tension (g.)
		Acetylcholine				
1	10^{-7}	45	195	—	3·9	3·4
	10^{-7}	45	195	FG	4·3	3·2
	10^{-7}	45	195	FG	7·5	3·3
	10^{-5}	120	60		0·5	5·0
	10^{-7}	45	135	DE	18·0	2·1
	10^{-7}	45	135	FG	5·7	3·1
	10^{-7}	45	135	FG	3·8	3·5
	10^{-7}	45		HI	2·0	4·0
2	10^{-7}	45	195	—	9·5	2·3
	10^{-7}	45	195	DE	10·0	3·2
	10^{-5}	90	150		0·25	4·6
	10^{-7}	45	195	DE	38·0	1·5
	10^{-7}	45	195	DE	32·0	2·5
	10^{-7}	45		EF	7·0	2·8
		Histamine				
3	10^{-7}	60	180	—	30·5	1·2
	10^{-7}	60	180	FG	26·5	1·5
	10^{-5}	60	180	FG	5·0	2·7
	10^{-7}	60	180	EF	32·0	1·3
	10^{-7}	60	180	EF	27·5	1·3
	10^{-5}	120	120	FG	5·0	3·0
	10^{-7}	60		DE	44·0	1·0
4	10^{-7}	60	150	—	4·3	2·2
	10^{-5}	60	150		2·1	4·6
	10^{-7}	60		DE	33·0	1·2

TABLE II—*continued*

Expt. No.	Conc.	Duration of Exposure (sec.)	Duration of Interval (sec.)	Stage of Oscillation	Lag (sec.)	Tension (g.)
5	10^{-7}	60	150	—	18·0	2·2
	10^{-5}	60	210		6·2	4·0
	10^{-7}	60		FG	10·0	2·2
		5-Hydroxytryptamine				
6	2×10^{-9}	150	150	—	19·6	1·8
	2×10^{-9}	150	150	DE	28·0	1·6
	2×10^{-9}	150	150	DE	35·0	1·3
	2×10^{-9}	150		EF	55·0	0·9
7	5×10^{-8}	60	180	—	2·0	3·5
	5×10^{-8}	60	180	EF	10·5	2·3
	5×10^{-8}	60		EF	30·0	2·2
8	5×10^{-8}	45	195	—	14·6	1·0
	5×10^{-8}	45	195	FG	11·9	1·1
	5×10^{-8}	45	195	FG	16·2	0·9
	5×10^{-8}	90	150	N	33·0	0·7
	5×10^{-8}	45	240	EF	—	0
	5×10^{-8}	45	195	EF	4·0	1·5
	5×10^{-8}	45	150	FG	9·5	1·6
	5×10^{-8}	45		FG	13·5	1·7

We found that a reduction of the mechanical response to acetylcholine occurred most readily if a large dose was interposed in a series of smaller test doses. This was the condition described by Cantoni and Eastman (1946) and by Rand (1957). From our electrical records it appeared that, in order to observe this effect, the small dose had to be given during the period DE or near the time of maximal polarization. The fast rate at which the membrane repolarized after the removal of a large dose of acetylcholine delayed the response to a subsequent small dose considerably and reduced the mechanical response. (This is illus-

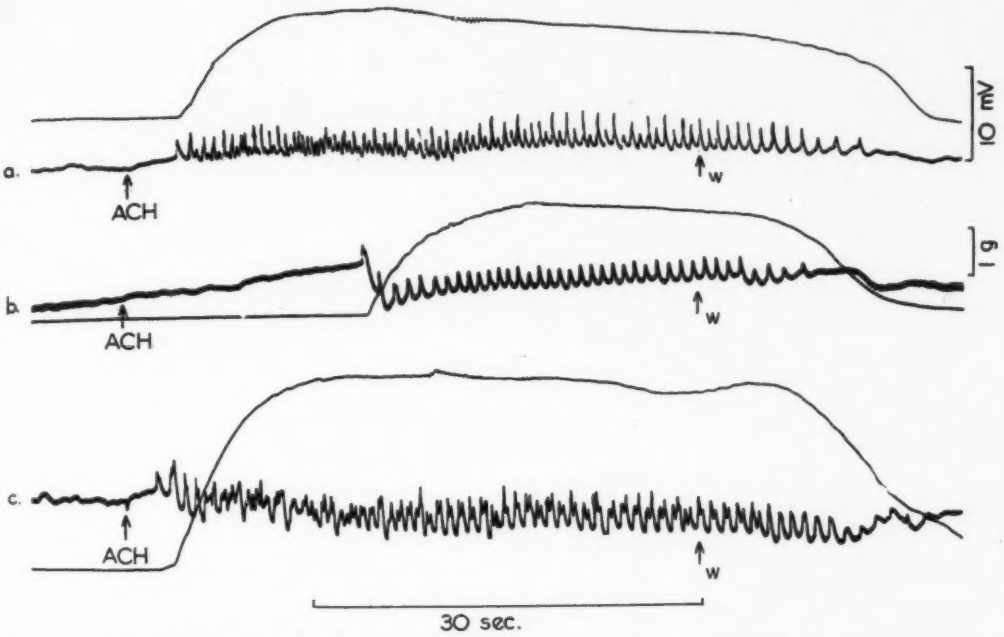

FIG. 5.—Tachyphylaxis produced by acetylcholine (ACH). Records as in Fig. 2*b*. Effects of acetylcholine 10^{-7}: (*a*) Control before exposure for 2 min. to acetylcholine 10^{-5}; (*b*) 1 min. after removal of the high concentration, when the membrane potential was 5 mV higher than in (*a*); and (*c*) 9 min. later when the membrane potential was 5 mV lower than in (*a*). Note longer lag in *b*, shorter lag in *c*. For description see text.

trated by Expts. 1 and 2 in Table II, and in Figs. 5 and 8.) On the other hand, the slow rate of repolarization following the removal of a weak concentration of acetylcholine might still prolong the lag of the next response, but the delay was insufficient to impair the production of tension. Fig. 5 shows three records from a series of responses to acetylcholine 10^{-7}: (*a*) is one of the controls obtained before, and (*b*) is the first response obtained after the removal of acetylcholine 10^{-5} at the time when the membrane was hyperpolarized with respect to the control. The lag was prolonged and the tension response was reduced; (*c*) is the fourth response obtained during a phase of the recovery oscillations when the membrane was depolarized with respect to the control. The lag was shortened and a greater tension was produced. This will be referred to later.

As the rate of depolarization due to histamine was slow, tachyphylaxis readily occurred even with repeated application of low doses, particularly if the exposure was somewhat longer or if the intervals between doses were short. For example, an interval of only 2 min. prolonged the lag while an interval of 3 min. shortened it (see Fig. 8*b*). Again tachyphylaxis was most severe when the test dose was applied during period DE, especially if a high concentration of histamine was interposed. This affected the responses differently according to the time of exposure and the interval until the test dose was applied, as shown by Expts. 3, 4, and 5 in Table II. Fig. 6 shows typical records of (*a*) the response to histamine 10^{-7} and (*b*) to histamine 10^{-5} applied for 1 min. After removal of the high concentration, while the membrane was rapidly repolarizing, the application of histamine 10^{-7} was repeated (*c*). Two spikes were seen, but further discharge stopped as the membrane continued to hyperpolarize. Indeed, a burst of spikes and the associated production of tension barely appeared before the histamine was due to be washed out. It can also be seen that, when the spikes finally appeared, the membrane polarization was still increasing, that is, it was moving in a direction which effectively counteracted depolarization and the response was diminished. This divorce of initiation of spike activity from membrane depolarization was characteristic of tachyphylaxis (it was especially pronounced with 5-hydroxytryptamine).

From the experiments described so far there appeared to be a clear relation between the point

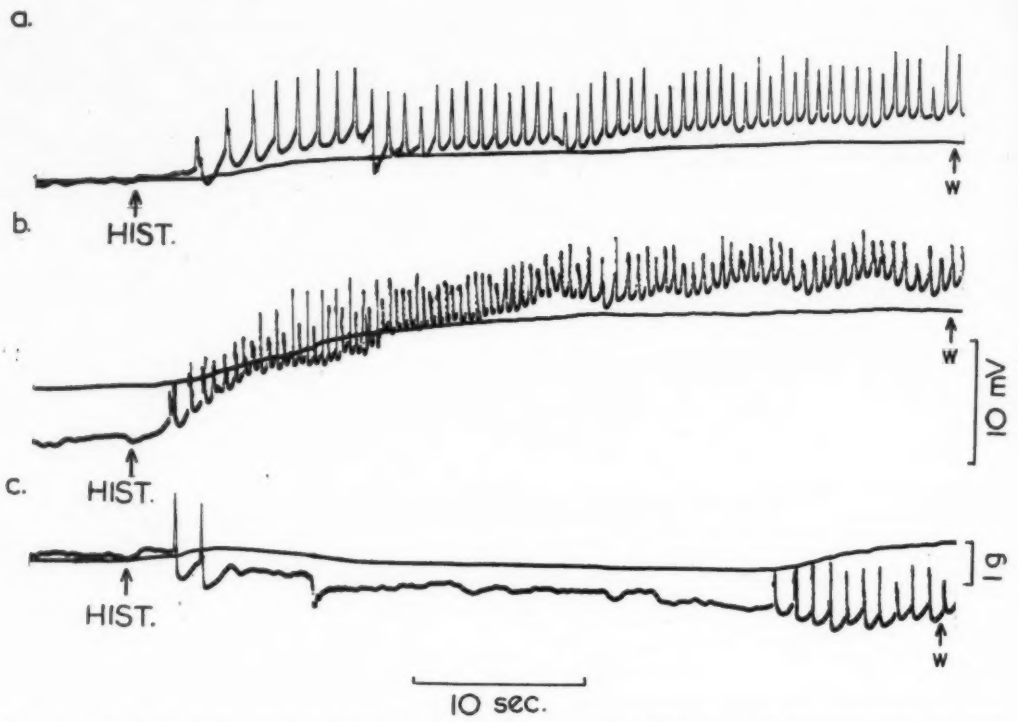

FIG. 6.—Tachyphylaxis produced by histamine. Records as in Fig. 2*b*. (*a*) Control 10^{-7}, (*b*) 10^{-5}, (*c*) 10^{-7}. The interval between the removal (*W*) and the next exposure to histamine was 2.5 min. each time.

during the recovery oscillations at which the application of a stimulating drug was repeated and the degree of tachyphylaxis which was observed. This relation held for 5-hydroxytryptamine only if low doses were applied for short periods. As the maximum response time for 5-hydroxytryptamine was, however, longer than 1 min., it had to be applied for longer periods if the full effect was to be obtained. It was then observed that tachyphylaxis occurred in almost every instance. An example is shown in Fig. 7. Three experiments in which different periods of exposure and different intervals between doses were used are shown in Table II. If the duration of application was longer, or the intervals shorter, or when stronger concentrations were used, the conditions became complicated. The degree of tachyphylaxis was no longer correlated with the membrane oscillations. It was also found that oscillations occurred already in the presence of 5-hydroxytryptamine and persisted, often accentuated, after its removal, so that it was impossible to decide whether successive doses produced an effect of their own or whether they merely coincided with the oscillation.

Potentiation

Potentiation by Repeated Application of the Same Drug.—In many experiments it was observed that the response to a weak concentration of acetylcholine, after having been impaired by a strong concentration, was subsequently potentiated. This is illustrated in Fig. 5*c*. The suppression of the first response after the strong concentration, as recorded in (*b*), effectively increased the interval between doses. Furthermore the response recorded in (*c*) occurred in the phase FG, when the membrane was depolarized. Another experiment of this kind is shown graphically in Fig. 8*a*, in which the depression of the response during the phase DE was followed later by a potentiation of the response during phase EF.

Potentiation occurred also with histamine when weak concentrations were applied repeatedly. Fig. 8*b* shows a graph of an experiment in which histamine 10^{-7} was used. It can be seen that the second dose, applied at E, produced a response with a prolonged lag but an unimpaired tension. The third dose, applied after a longer interval, so that it was introduced during the phase FG, had a shorter lag and produced a greater tension

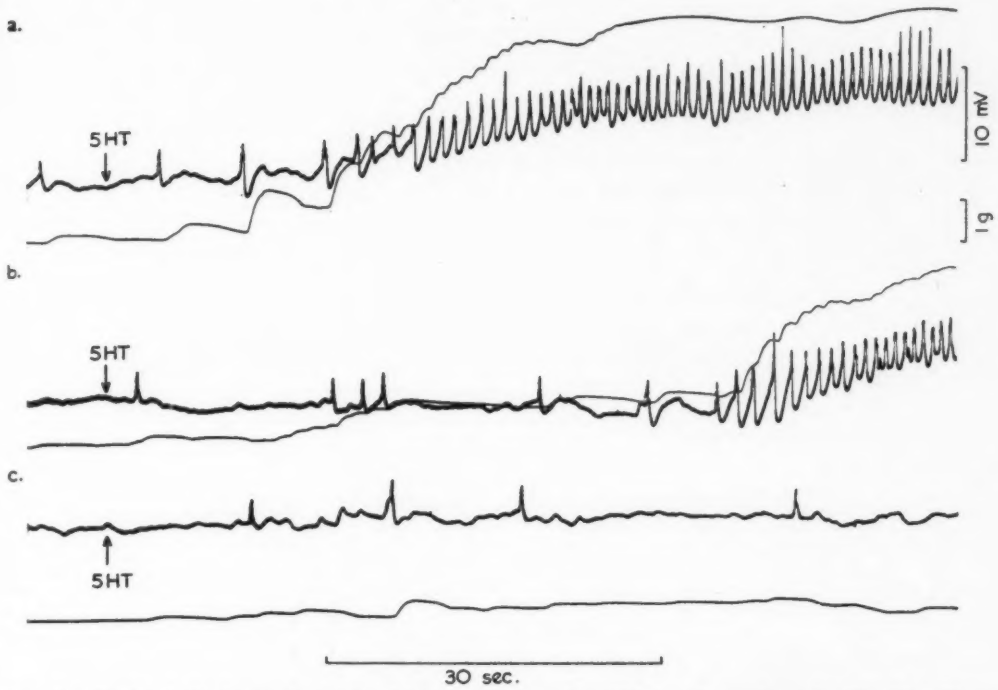

FIG. 7.—Tachyphylaxis produced by 5-hydroxytryptamine (5HT). Records as in Fig. 2*b*. Three successive applications of 5-hydroxytryptamine 5×10^{-8} applied for 2 min. every 4 min. Note the increased lag in (*b*) compared with (*a*) and the absence of any effect in (*c*).

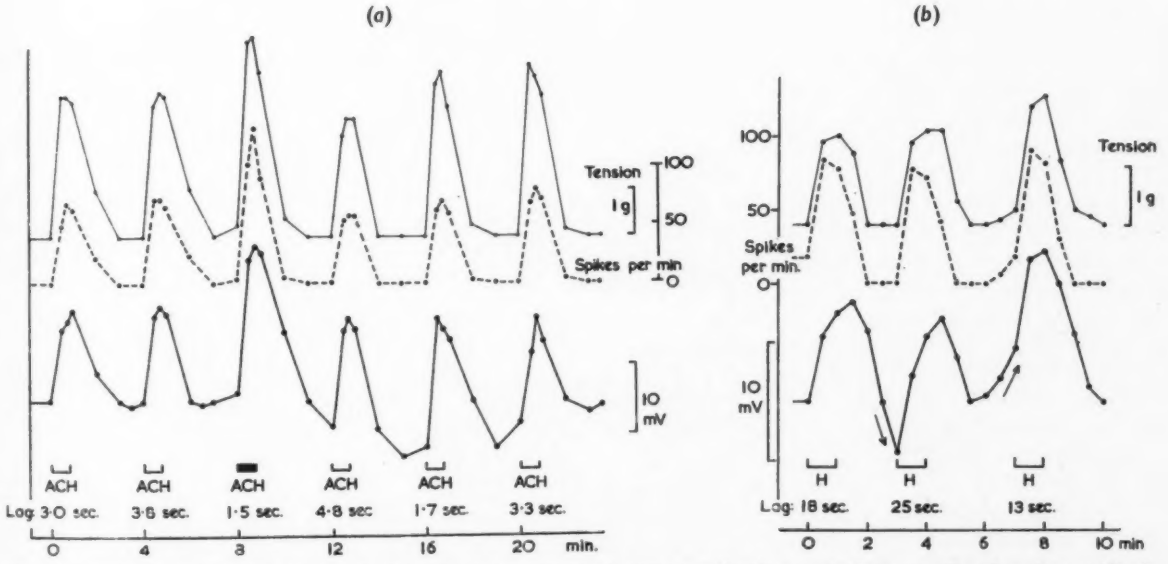

FIG. 8.—Potentiation following tachyphylaxis after repeated drug application. Ordinates: Tension (upper line); spike frequency (broken line); membrane potential (lower line). Abscissa: time in min. In (*a*) acetylcholine (ACH) 10^{-7} was given repeatedly, but once acetylcholine 10^{-5} (black bar) was interposed. In (*b*) the first interval between doses of histamine 10^{-7} was 2 min., the second interval 3 min. Note longer lag when drug was applied while membrane polarized, and shorter lag when drug was applied while membrane depolarized. For detailed description see text.

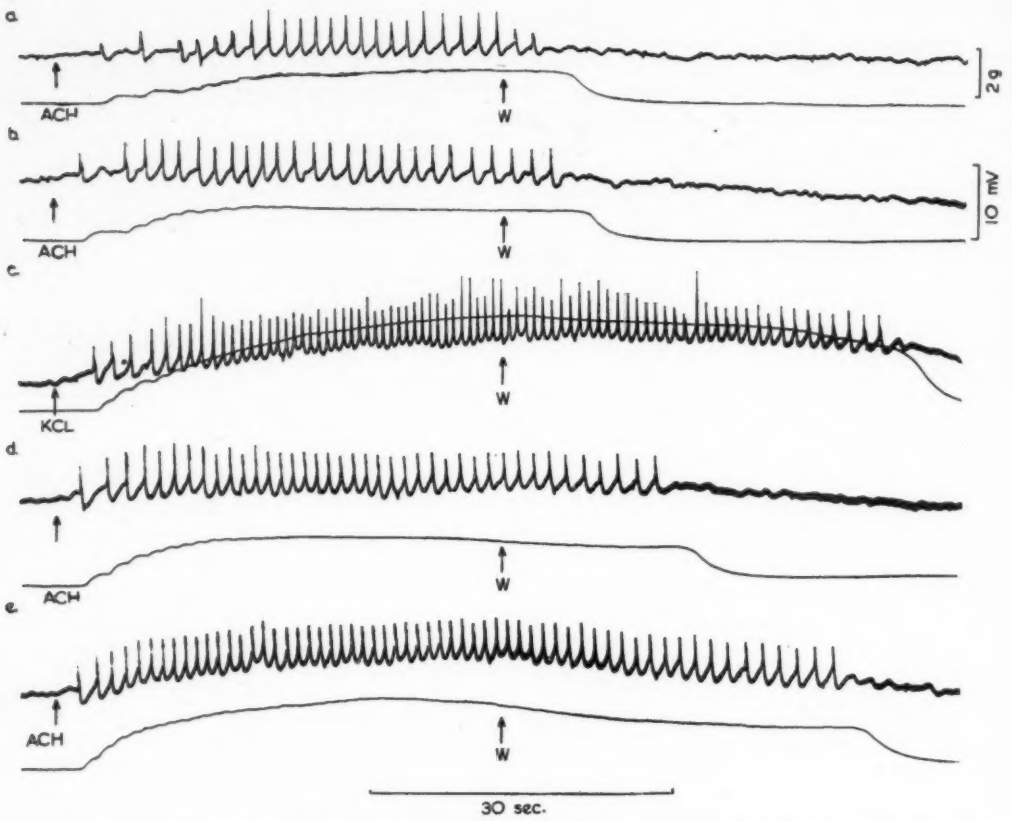

FIG. 9.—Potentiation of acetylcholine (ACH) effect after exposure to 20 mM potassium chloride (KCl). Records as in Fig. 2*b*. Doses were applied for 45 sec. every 4 min.; (*a*), (*b*), (*d*), and (*e*) acetylcholine 10^{-7}, (*c*) 20 mM potassium chloride. In (*d*) the membrane was depolarized by 5 mV, in (*e*) by 4 mV, with respect to the initial potential.

change. With 5-hydroxytryptamine we observed potentiation only in one experiment.

Potentiation after KCl.—Cantoni and Eastman (1946) observed that the interposition of a strong concentration of potassium chloride in a series of small doses of histamine or acetylcholine did not depress the response but enhanced it. It has been shown by Burnstock and Straub (1958) and by Holman (1958) that exposure to 20 mM potassium chloride caused a depolarization of 16 to 20 mV, and that after washing in normal solution the membrane potential slowly returned to the initial value over a period of several minutes, during which time there was no phase of hyperpolarization. We have applied acetylcholine or histamine during the recovery from exposure to potassium chloride while the membrane was still depolarized. We always found an augmentation of the response (Cantoni and

TABLE III

EFFECT OF 5-HYDROXYTRYPTAMINE ON THE RESPONSE TO ACETYLCHOLINE

Expt. No.	Conc. of Acetylcholine	Control		In the Presence of 5-Hydroxytryptamine (5×10^{-8})		1 min. after Removal of the 5-Hydroxytryptamine	
		Lag (sec.)	Tension (g.)	Lag (sec.)	Tension (g.)	Lag (sec.)	Tension (g.)
1	10^{-7}	16·2	1·1	4·0	2·7	—	
2	10^{-6}	4·7	2·9	0·5	4·6	—	
3	10^{-6}	12·4	1·5	—		0·7	1·8
4	10^{-6}	5·5	2·6	—		4·3	3·2

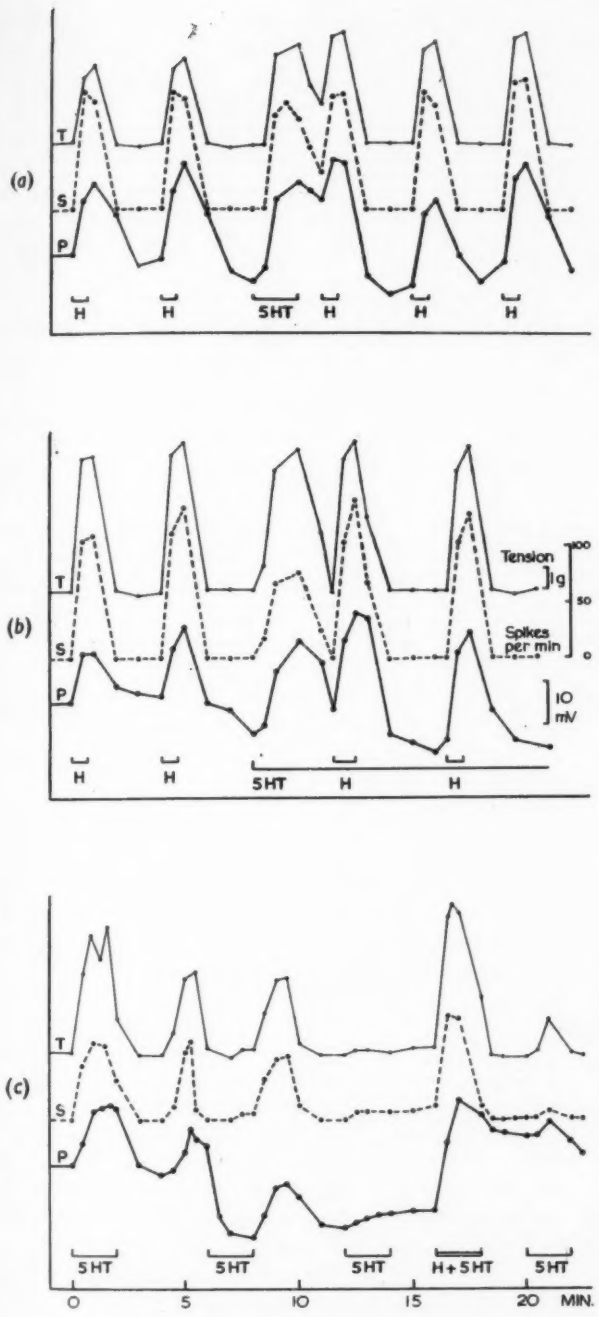

FIG. 10.—Potentiation of the effect of histamine (H) by 5-hydroxytryptamine (5HT). Symbols as in Fig. 8. The response to a constant dose of histamine (10^{-7}) was enhanced (*a*) by the interposition of a dose of 5×10^{-8} of 5-hydroxytryptamine. It was further enhanced (*b*) in the presence of 5-hydroxytryptamine, and still larger (*c*) though the muscle had been desensitized to 5-hydroxytryptamine.

Eastman, 1946; Rand, 1957). This augmentation was associated with a shorter lag. A typical experiment is shown in Fig. 9.

Potentiation of the Acetylcholine and Histamine Response by 5-Hydroxytryptamine.—The responses to acetylcholine and to histamine were potentiated by 5-hydroxytryptamine (Bülbring and Crema, 1958; Draškoci and Haranath, 1959). This potentiation was independent of the time of application during recovery from the 5-hydroxytryptamine, and was also seen in the presence of 5-hydroxytryptamine (see Table III).

Fig. 10 shows an experiment in which the potentiation of the response to histamine by 5-hydroxytryptamine was demonstrated in three different ways. In (*a*) the response to histamine was increased by interposition of 5-hydroxytryptamine. The potentiation was still evident 13 min. later (*b*) and was undiminished in the presence of 5-hydroxytryptamine. In (*c*) the preparation was desensitized to 5-hydroxytryptamine by repeated exposure. Yet, when histamine was applied together with 5-hydroxytryptamine, its effect was greater still.

DISCUSSION

The depolarization which occurs during exposure of intestinal smooth muscle to a stimulating drug is followed, after its removal, by a phase of hyperpolarization and then by a period during which the membrane potential fluctuates. This sequence of events had been noticed by Bülbring (1955) using intracellular recording, but in the present work it could be followed more accurately by continuous recording using the sucrose gap technique. Recent evidence (Burnstock, 1958a) suggested that the depolarization caused by acetylcholine (and possibly by other stimulating drugs) might be the result of a simultaneous increase in permeability to K^+, Na^+, and perhaps other ions. The observations of Durbin and Jenkinson (1959), who found that carbachol increased the permeability of the smooth muscle membrane to both potassium and chloride, are consistent with this view.

The events which follow during the recovery may be understood after considering some recent observations concerning the factors which determine the excitability of the taenia coli. It appears that the membrane permeability to sodium is much greater than that of other excitable tissues (Goodford and Hermansen, 1960). This may be

one of the reasons for the tendency to fire spontaneously and for the relatively low membrane potential. The rate of energy supply to the membrane would therefore be expected to be of great importance for the maintenance of membrane polarization, as energy is required for active ion transport (Hodgkin and Keynes, 1955) and probably also for the reduction of the inward leakage of sodium (Shanes, 1958). In taenia coli metabolic inhibitors cause depolarization and increase the spontaneous discharge (Born and Bülbring, 1955; Bülbring and Lüllmann, 1957) as well as excitability (Axelsson and Bülbring, unpublished observations). Removal of glucose from the medium or sudden cooling, for example from 37° to 26°, has the same effect (Axelsson and Bülbring, 1960a and b). On the other hand, a rise in temperature after a period of cooling, or the restoration of the normal glucose concentration after a period of glucose depletion, stabilizes the membrane. The cessation of spontaneous discharge, the loss of excitability and the hyperpolarization produced by a sudden increase of metabolic rate are phenomena which are also characteristic for the recovery after drug stimulation and, to a larger extent, for the effect produced by adrenaline. Axelsson, Bueding and Bülbring (1959) have recently shown that the inhibitory effect of adrenaline coincides with an activation of phosphorylase. This stimulation of one reaction in a chain of metabolic processes supplying energy is likely to have a causal connexion with the membrane stabilization. Born and Bülbring (1956) observed an increased rate of potassium uptake in the presence of adrenaline, which was similar to that observed after the removal of stimulating drugs. They concluded that adrenaline accelerated active processes in the cell membrane which operate also normally during recovery from activity. Burnstock (1958b) suggested that the hyperpolarization produced by adrenaline might be due to a transitory shift in the balance of ionic fluxes through the membrane involving an electrogenic extrusion of sodium. There is evidence (Bülbring and Goodford, unpublished observations) that adrenaline reduces the rate of uptake and increases the rate of loss of radioactive sodium in taenia coli.

In the experiments described in the present paper we observe a transient depression of excitability during those phases of the recovery period when the polarization of the membrane is increasing. We may assume that the loss of excitability is associated with an accelerated energy supply to the membrane causing an acceleration of the sodium pump and possibly a reduction of the inward leakage of sodium. However, as soon as the membrane becomes hyperpolarized—or rather when the potential approaches its true resting level—the rate of active processes would slow. This, with the relatively high membrane permeability to sodium, would lead to depolarization which, in turn, would provoke a repetition of the initial process. The oscillations of excitability and of membrane polarization can then be explained as being due to a periodically changing rate of active processes at the membrane affecting its stability and causing periodical shifts in the balance of ion fluxes. It may well be that also in normal conditions in which we find spontaneous fluctuations of the membrane potential a cycle of the type described operates, in which metabolic rate and excitability are linked (see Bozler, 1948).

The action of the three stimulating drugs, acetylcholine, histamine and 5-hydroxytryptamine, was studied by applying them for relatively short periods and at short intervals, that is, in conditions which might prevail during a biological assay. The magnitude of the response to repeated doses was found to depend on the time of application during the recovery oscillations of the membrane potential which followed the previous dose. During the first period of hyperpolarization, while the membrane potential was actually increasing (DE), tachyphylaxis was most pronounced. But, as a result of the differences in the rates of depolarization, which was fastest with acetylcholine, tachyphylaxis was less readily observed with small doses of acetylcholine than with histamine and 5-hydroxytryptamine. However, if a small dose of acetylcholine was given immediately after a large dose, tachyphylaxis was readily seen. In this condition the rate of repolarization following removal of a high concentration was equal or faster than the rate of depolarization caused by the weak concentration; and thus the response to the small dose was reduced. During the subsequent fluctuations, if the drug was applied while the membrane was hyperpolarizing, the lag was prolonged; but if it was applied during phases of depolarization, the response was facilitated, that is, the lag was shorter. The mechanical effect of acetylcholine and histamine was then potentiated.

From these observations it became evident that the excitability of the tissue by the stimulating drug was not determined by the absolute value of the membrane potential but by the direction into which the potential moved and by the rate at which this occurred at the moment when the

drug was applied. Furthermore, whenever the membrane was in the process of depolarization the threshold at which spikes appeared was at a much higher absolute level of membrane potential than that at which they stopped when the membrane was in the process of repolarization (Fig. 2*b*), indicating that there was no absolute firing level. Thus the degree of tachyphylaxis depended on the ratio between the rate of the spontaneous change in membrane potential and the rate at which the drug depolarized. As this rate was fastest with acetylcholine, slower with histamine, and slowest with 5-hydroxytryptamine, tachyphylaxis occurred least with acetylcholine and more with the other two drugs.

The observation by Cantoni and Eastman (1946) and by Rand (1957) that pretreatment with excess potassium chloride enhanced the response to subsequent doses of acetylcholine and histamine falls into line with our interpretation. As the recovery of the membrane potential after exposure to excess potassium chloride consisted of a simple, slow return to the initial level (Burnstock and Straub, 1958; Holman, 1958), the membrane was still depolarized when the next dose of acetylcholine was applied. The decreased lag was therefore not surprising and the response to acetylcholine was enhanced.

It is possible to interpret the observation by Hughes, McDowall and Soliman (1956) along the same lines. They found that tachyphylaxis depended on the external sodium concentration. If this was high there would be a larger increase of the internal sodium concentration during exposure to the stimulating drug. This would cause a greater acceleration of the sodium pump (Hodgkin and Keynes, 1956) and in the light of the present hypothesis favour tachyphylaxis.

Our observations suggest that 5-hydroxytryptamine, unlike acetylcholine and histamine, produced some more permanent alteration of the membrane properties. Whereas, with acetylcholine and histamine, the larger the concentration applied (up to a limit) the greater was the rate of depolarization produced, this was not so for 5-hydroxytryptamine, where increasing concentrations caused a progressively slower rate of depolarization. Tachyphylaxis often occurred when 5-hydroxytryptamine was introduced at a stage during which acetylcholine and histamine responses would have been potentiated.

The changes of the membrane properties produced by 5-hydroxytryptamine may be the result of absorption of the non-ionized form of the molecule on to the membrane, thereby impairing the depolarizing action of the ionized form. Another explanation for the ready occurrence of tachyphylaxis to 5-hydroxytryptamine is provided by the finding of Born (unpublished) that 5-hydroxytryptamine is taken up by the smooth muscle cells.

The action of acetylcholine and histamine was potentiated both after treatment and in the presence of 5-hydroxytryptamine (see also Bülbring and Crema, 1958; Draškoci and Haranath, 1959). That acetylcholine and histamine were still active when the muscle had been made insensitive to 5-hydroxytryptamine could be explained partly by the fact that both acetylcholine and histamine depolarize at a much faster rate than 5-hydroxytryptamine. Secondly, they are likely to act on different receptors (Gaddum, 1953). The potentiation is, however, difficult to explain. It might be due to the fixation of 5-hydroxytryptamine within the tissue leading to a change in membrane properties.

With the exception of the specific effects produced by 5-hydroxytryptamine, the results described are consistent with the hypothesis that the response obtained by a stimulating drug, applied for short periods in rapid succession, depends firstly on the rate at which it depolarizes. Secondly, the response depends on the state of the membrane and its excitability. This appears to be determined by the fluctuating rates of active ion transport and of metabolism. Experiments are in progress to test this hypothesis further.

Our thanks are due to the Medical Research Council for a grant to one of us (G. B.). We also wish to thank Mr. O. B. Saxby and Mr. D. Groves for their technical assistance.

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A COMPARATIVE STUDY OF HYDROXYINDOLE OXIDASES

BY

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A comparative study has been carried out of the oxidation of 5-hydroxytryptamine and related compounds by the oxidase present in the gill plates of *Mytilus edulis* and of caeruloplasmin, the copper containing oxidase of mammalian plasma. Both preparations oxidized indole derivatives carrying a hydroxyl group in the 4-, 5-, 6-, or 7- position. The oxidation of bufotenine was compared with that of its 4- and 6-hydroxy analogues; the 4-hydroxy analogue is psilocine, a naturally occurring hallucinogenic compound. Bufotenine and the 6-hydroxy analogue were oxidized by both preparations with the formation of brown pigments; psilocine was more rapidly oxidized with the appearance of a blue colour. 4-Hydroxytryptamine and 7-hydroxytryptamine were also oxidized, the former with the formation of a blue compound. The N-1-methyl derivatives of both bufotenine and psilocine were also oxidized. The *Mytilus* preparation acted also on 4-, 5-, and 7-hydroxytryptophan and on 5-hydroxyindole, none of which was oxidized by caeruloplasmin. The *Mytilus* enzyme also oxidized 5-hydroxyindoleacetic acid. Paraphenylenediamine, a very good substrate of caeruloplasmin, was much more slowly oxidized by the gill plate enzyme. The evidence suggests that the two enzymes catalyse the same reactions, but that the substrate specificity of the mammalian oxidase is somewhat more restricted. Both enzymes are " hydroxyindole oxidases," not specific for 5-hydroxyindoles alone. Inhibitors of the *Mytilus* oxidase included inhibitors of copper enzymes but not edetate or carbon monoxide. The action of pig serum on 5-hydroxytryptamine was due to caeruloplasmin and not to amine oxidase.

The oxidation of 5-hydroxytryptamine by amine oxidase and the subsequent formation of 5-hydroxyindoleacetic acid are reactions that play an important part in the inactivation of 5-hydroxytryptamine in mammals (see Udenfriend, 1958; Blaschko, 1958). However, it is also known that other pathways of breakdown of 5-hydroxytryptamine must exist. Erspamer (1955) has shown that in man and dog less than one-half of the amine administered is excreted as 5-hydroxyindoleacetic acid in the urine, and he has found that in a number of herbivorous species the urine contains only insignificant amounts of 5-hydroxyindoleacetic acid. It is not known to what extent this deficit is accounted for by known metabolites of 5-hydroxytryptamine, for example, the O-glucuronate and the O-sulphate, the N-acetyl derivative or 5-hydroxyindoleaceturic acid (Curzon, 1957; Chadwick and Wilkinson, 1958; McIsaac and Page, 1959).

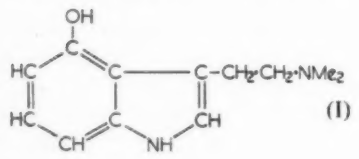
Little is known of the biological inactivation of 5-hydroxytryptamine in invertebrates. In molluscs, amine oxidase has been found in many species (Blaschko, Richter, and Schlossmann, 1937; Blaschko, 1941). Erspamer (1948) reported that enteramine was destroyed by the amine oxidase from Octopods, and after the identity of enteramine with 5-hydroxytryptamine had been established it was shown that the synthetic amine was oxidized by the molluscan enzyme (Blaschko, 1952a). In *Mytilus edulis* the digestive gland as well as the anterior retractor muscle of the byssus contain an amine oxidase that acts on 5-hydroxytryptamine (Blaschko and Hope, 1957).

In the gill plates of *Mytilus* there occurs another enzyme which oxidizes 5-hydroxytryptamine (Blaschko and Milton, 1959, 1960). This differs from amine oxidase. It acts also on other 5-hydroxyindoles, for example, 5-hydroxytryptophan and bufotenine. Unlike amine oxidase, this enzyme is cyanide-sensitive and it may be a kind of phenol oxidase, acting upon the indole ring with the formation of a quinone-imine, a reaction discussed some time ago (Blaschko and Philpot,

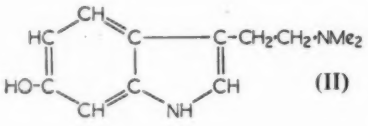
1953). Support for this formulation is seen in two observations: first, a brown pigment is formed in the enzymic reaction and, second, the gill plate preparation, even when somewhat purified, is still able to oxidize catechol derivatives, for example, dopa or dopamine.

It seemed probable that catalysts analogous to the gill plate enzyme occur in mammals. Caeruloplasmin, the copper containing protein of mammalian plasma, has been reported to act on 5-hydroxytryptamine (Porter, Titus, Sanders and Smith, 1957; Martin, Eriksen and Benditt, 1958; Nakajima and Thuillier, 1958; Siva Sankar, 1959; Zarafonetis and Kalas, 1960; Curzon and Vallet, 1960). Since caeruloplasmin is also able to oxidize catechol compounds and, as a copper enzyme, is cyanide-sensitive, it was decided to compare the action of the oxidase from *Mytilus* gill plates with that of pig plasma caeruloplasmin.

In order to obtain more information on the reaction catalysed by the two enzymes, the study has been extended to indole derivatives other than those carrying a hydroxyl group in position 5. The compounds tested were all hydroxyindoles. They include the 4-hydroxy and the 6-hydroxy analogue of bufotenine (I and II) and two *N*-methyl derivatives, that of bufotenine (III) and the corresponding 4-hydroxy compound (IV). In addition, 4-hydroxytryptamine, 7-hydroxytryptamine, 4-hydroxytryptophan, and 7-hydroxytryptophan were tested. The formula of 7-hydroxytryptamine is shown in (V).



(I)



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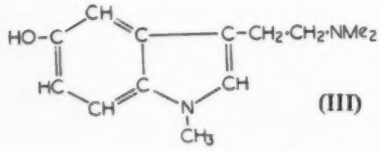

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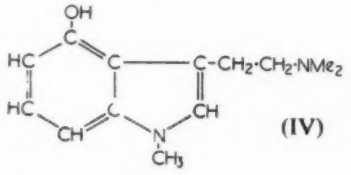
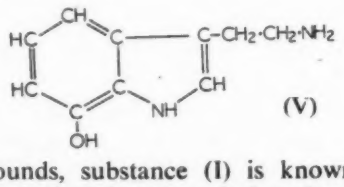


(IV)



(V)

Of these compounds, substance (I) is known to be of pharmacological interest; it has been identified with psilocine, a hallucinogenic substance occurring in the fungus, *Psilocybe mexicana Heim* (Hofmann and Troxler, 1959; Hofmann, Heim, Brack, Kobel, Frey, Ott, Petrzilka and Troxler, 1959).

Methods

Enzyme Preparations.—The *Mytilus* gill plate preparations used were similar to those described by Blaschko and Milton (1960). Homogenates of the gill plates were prepared and the supernatant fluid, after centrifugation for 2 hr. at 5,000 *g*, was fractionated with ammonium sulphate, the material precipitating between 30 and 60% saturation being rich in oxidase activity. The precipitate was dissolved and dialysed first against running tap water and then in the cold room against distilled water. The dialysed preparation was freed from a slight precipitate by centrifugation for 30 min., at 100,000 *g*, and the supernatant fluid was used.

Caeruloplasmin was prepared from pig serum according to Holmberg and Laurell (1948). Material precipitating between 35 and 50% saturation with ammonium sulphate was collected and dialysed against running tap water overnight. Impurities were removed by two successive centrifugations, the first after adjusting the *p*H to 6.2 with 5% acetic acid, the second after adjustment to *p*H 5.5. The caeruloplasmin was then precipitated in 15% ethanol at 0°. The precipitate was resuspended in 0.9% sodium chloride, and after dialysis overnight the *p*H was adjusted to 6.5 and an equal volume of an ethanol-chloroform (9:1, v/v) mixture was added. The resulting precipitate was extracted with saline until the washings no longer had the blue colour characteristic of caeruloplasmin. The second ethanol-chloroform treatment used by Holmberg and Laurell (1948) was omitted. The extracts were combined and concentrated by precipitation with ammonium sulphate at 65% saturation. Dialysis against tap water overnight was followed by centrifugation for 30 min. at 5,000 *g*, in order to remove a turbid contaminant.

Measurement of Enzyme Activity. — Oxygen uptake was measured manometrically. The temperature of the manometer bath was 37.5°, with both the molluscan and mammalian preparations. The gas phase was air. The substrate concentrations were 5×10^{-3} M for the indole derivatives and 10^{-2} M for paraphenylenediamine. In the experiments with the *Mytilus* oxidase, 0.067 M sodium phosphate buffer of *p*H 7.4 was used. With caeruloplasmin, the *p*H of the buffer was 6.0; in the experiments with the indole derivatives 0.067 M sodium phosphate buffer was used, in experiments with paraphenylenediamine

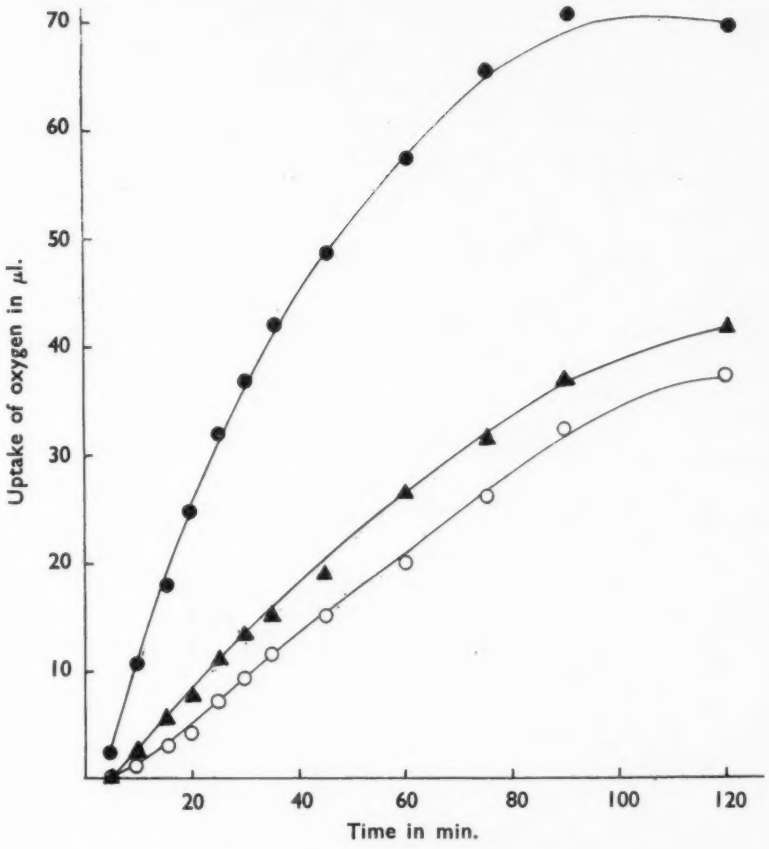


FIG. 1.—The rate of oxidation of bufotenine (O—O), psilocine (●—●), and 6-hydroxy-*N*-*N*-dimethyltryptamine (▲—▲) by *Mytilus* gill plate oxidase. The enzyme was incubated at 37.5° with 5×10^{-3} M substrate in phosphate buffer at *p*H 7.4. The gas phase was air.

0.01 M acetate buffer was used. The substrates were tipped in from the side arms of the conical manometer flasks.

Substances Used.—The 5-hydroxyindole was prepared by Dr. E. W. Gill, of this department, by catalytically debenzylating 5-benzyloxyindole (Ek and Witkop, 1954); it was purified by sublimation and recrystallization. The bufotenine was prepared by Dr. R. B. Barlow, of Edinburgh University; this was the sample used by Blaschko and Milton (1960). Compounds I, II, III and IV were given to us by Dr. A. Hofmann, of Sandoz A.-G., Basel.

Professor V. Erspamer, Parma, kindly gave us samples of 4-hydroxytryptamine creatinine sulphate, 7-hydroxytryptamine hydrochloride, 4-hydroxytryptophan, and 7-hydroxytryptophan; all these substances were prepared in the laboratories of Farmitalia, Milan.

We should like to record our gratitude for these valuable gifts.

RESULTS

Observations on the Mytilus Gill Plate Oxidase: Substrate Specificity

The observation that bufotenine is a substrate of the gill plate enzyme was readily confirmed. Bufotenine was therefore used together with the other indole derivatives. Fig. 1 shows an experiment in which the oxidation of bufotenine was compared with that of its 4-hydroxy and 6-hydroxy analogues. The first of these is psilocine. It can be seen that the rate of oxidation of the 6-hydroxy analogue was similar to that of bufotenine; psilocine was oxidized more rapidly. The experiment was terminated after 2 hr. when the uptake of oxygen with psilocine had ceased; with the 5- and 6-hydroxy analogues it still continued at a slow rate. The total uptake with psilocine was 70 μl. O_2; this

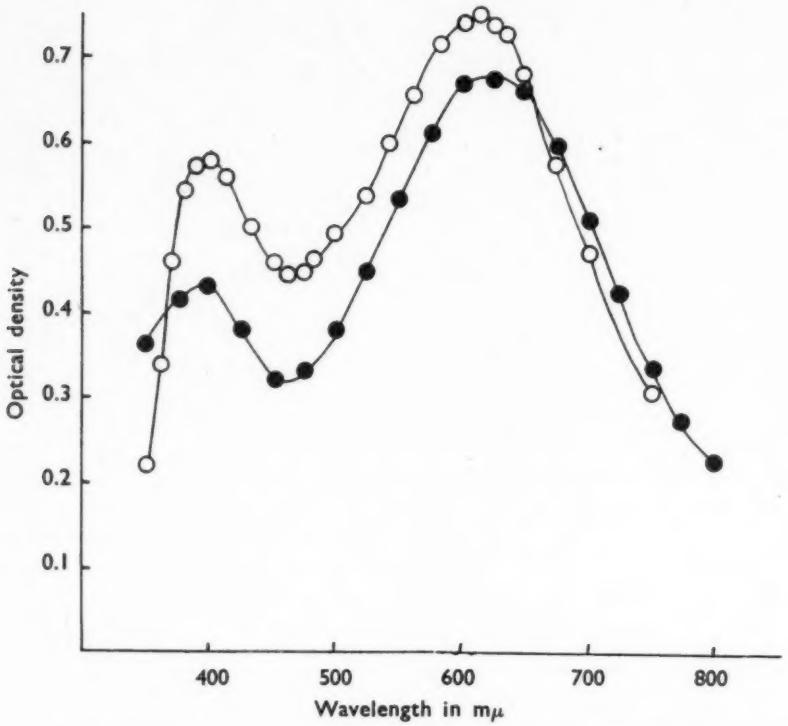


FIG. 2.—Absorption spectra of pigments formed in the oxidation of psilocine by the *Mytilus* enzyme (●—●) and by ferric sulphate (O—O).

represents 1.25 atoms of oxygen/mole of psilocine added. For 5-hydroxytryptamine, the total oxygen uptake is 0.9 atoms/mole of substrate (Blaschko and Milton, 1960).

During the oxidation of these three compounds, coloured products were formed. As observed by Blaschko and Milton (1960), the flask containing bufotenine acquired a brownish colour. With the 6-hydroxy analogue, a similar yellowish-brown colour developed. In the flask containing psilocine a deep blue colour became apparent. After centrifugation the blue pigment from psilocine remained in the supernatant fluid; any colour deposited with the sediment could be brought into solution by adding a few drops of N hydrochloric acid. The brown pigments formed from the other two isomers, on the other hand, remained entirely in the sediment and could not be similarly eluted.

The absorption spectrum of the blue pigment obtained from psilocine is shown in Fig. 2. The curve shows absorption maxima at 620 and at 400 mμ. That the blue material was an oxidation product was made likely by the observation that the colour was bleached upon adding sodium bisulphite, a reducing agent. Also, a blue compound was formed when a solution of psilocine in sodium phosphate buffer of *p*H 7.4 was oxidized by adding ferric sulphate. The time course of the development of the blue colour was followed spectrophotometrically by measuring the increase in absorption at 625 mμ. The reaction was complete within 20 min. The absorption was then measured throughout the visible range (see Fig. 2); the spectrum obtained was similar to that of the material formed when psilocine was oxidized by the gill plate enzyme, except for a shift of the absorption maximum at 620 mμ to 610 mμ and a relative increase of the peak at 400 mμ.

The *N*-methyl derivatives of both bufotenine and psilocine (III and IV) were also tested. They were also oxidized by the *Mytilus* oxidase. A comparison of the oxidation of the two *N*-methyl derivatives with that of the two parent compounds is shown in Fig. 3. Methylation of the ring nitrogen of psilocine depressed the initial rate of oxidation by 40%, but the rate of oxidation of *N*-methylbufotenine was about 30% higher than that of bufotenine. The oxidation product of *N*-methylpsilocine had a distinctly more greenish-blue hue, as compared to the deep

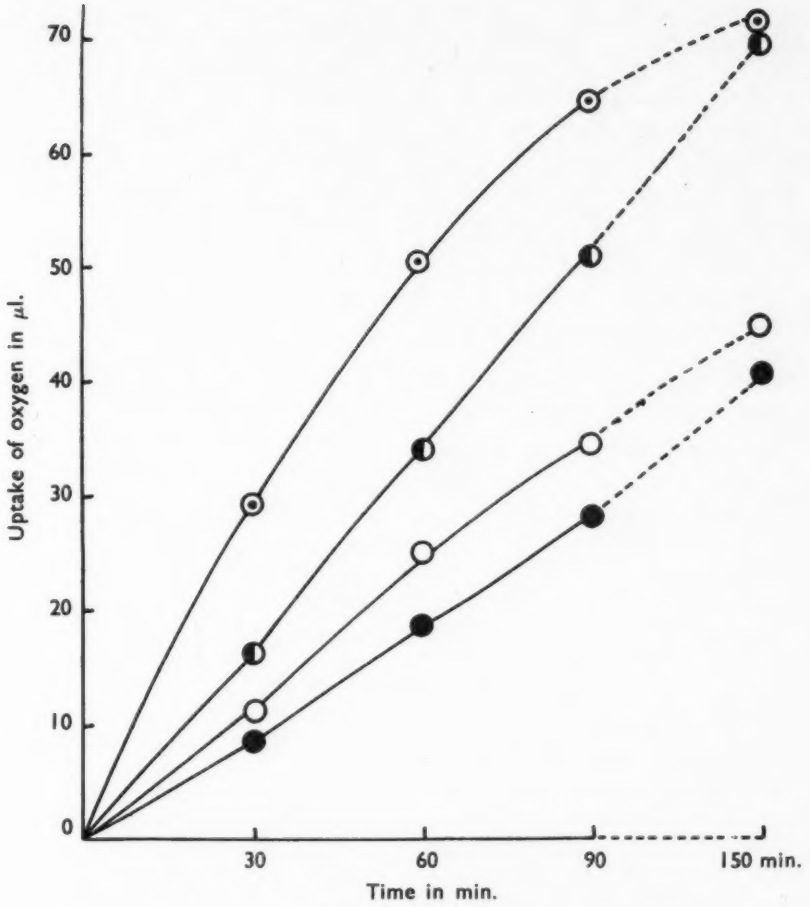


FIG. 3.—The rate of oxidation of bufotenine (●—●—●), psilocine (⊙—⊙), N-methylbufotenine (O—O), and N-methylpsilocine (◐—◐) by the *Mytilus* gill plate preparation. The enzyme was incubated at 37.5° with 5×10^{-3} M. Substrate in phosphate buffer at *p*H 7.4. The gas phase was air.

blue colour when psilocine was used as the substrate. This difference in colour is reflected in a difference in the absorption spectrum. The ratio, absorption at 400 mμ: absorption at 620 mμ, was increased from 0.685 for the oxidized psilocine to 1.14 for the oxidized N-1-methylpsilocine.

The gill plate preparation also oxidized 4- and 7-hydroxytryptamine as well as 4- and 7-hydroxytryptophan. In presence of one of the two 4-hydroxy compounds, oxygen uptake was rapid and was accompanied by appearance of a blue colour, similar to that of the oxidation product of psilocine. The oxidation of the two 7-hydroxy compounds was less rapid, being about equal to the rate of oxidation of 5-hydroxytryptamine; a brown pigment was formed when the 7-hydroxy compounds were oxidized.

In order to establish the role of the side chain in the enzymic oxidation, 5-hydroxyindole was also tested. It was oxidized, and its rate of oxidation was very similar to that of 5-hydroxytryptamine. However, in contrast to the brown pigment formed during the oxidation of 5-hydroxytryptamine, the product of oxidation of 5-hydroxyindole had a yellow-green colour.

Another hydroxyindole tested was 5-hydroxyindoleacetic acid. It was also rapidly oxidized by the gill plate enzyme, at about the same rate as 5-hydroxytryptamine. It is known that 5-hydroxyindoleacetic acid is oxidized by human caeruloplasmin (Curzon and Vallet, 1960).

Paraphenylenediamine was also oxidized by the gill plate preparation but at a rate only slightly greater than 5-hydroxytryptamine. This was in

sharp contrast to the more rapid rate of oxidation of paraphenylenediamine by caeruloplasmin.

Inhibitors of the Mytilus Oxidase

It has already been reported that the gill plate enzyme is cyanide-sensitive (Blaschko and Milton, 1960). Other substances that were tested as inhibitors of the oxidation of 5-hydroxytryptamine are listed in Table I. No inhibition was seen with carbon monoxide and with 10^{-2} M edetate. It is of interest that not all copper containing enzymes are inhibited by carbon monoxide. Keilin and Mann (1939) found no inhibition of laccase; caeruloplasmin is also not inhibited (Holmberg and Laurell, 1951a).

TABLE I

INHIBITORS OF THE HYDROXYINDOLE OXIDASE OF *MYTILUS* GILL PLATES

Substrate 5×10^{-3} M 5-hydroxytryptamine.

Inhibitor	Concentration	% Inhibition
Sodium diethyldithiocarbamate ..	10^{-3} M	47
Sodium azide ..	10^{-2} M	88
Salicylaldoxime ..	10^{-2} M	27
Diquinolyl ..	10^{-3} M	91
Edetate	10^{-2} M	0
Carbon monoxide	80% CO + 20% O_2	0

Observations on Caeruloplasmin of Pig Plasma

Under the conditions of our experiments, 5-hydroxytryptamine was oxidized by the preparations of caeruloplasmin. Geller, Eiduson and Yuwiler (1959) did not see any oxidation of 5-hydroxytryptamine by caeruloplasmin, but observations of Porter *et al.* (1957) and other authors already quoted are in agreement with our findings. Using 10^{-2} M paraphenylenediamine as standard, the relative initial rate of oxidation of 5-hydroxytryptamine was one-sixtieth of that of paraphenylenediamine. It may be mentioned that this refers to experiments in which the 5-hydroxytryptamine was oxidized in phosphate buffer of *p*H 6.0; its rate of oxidation was less in acetate buffer of the same *p*H. The reverse is true for paraphenylenediamine: its oxidation is far more rapid in acetate than in phosphate buffer (Holmberg and Laurell, 1951b).

Oxidation of 5-hydroxytryptamine was accompanied by the appearance of a brown pigment, similar to what was seen when the amine was oxidized by the preparation from *Mytilus* gill plates.

The pig plasma preparation also oxidized bufotenine and its 6-hydroxy and 4-hydroxy analogues. Psilocine was oxidized more rapidly, bufotenine more slowly, than 5-hydroxytryptamine. The 6-hydroxy analogue was oxidized at about twice the rate of 5-hydroxytryptamine; this is different from what was observed with the *Mytilus* enzyme.

Brown pigments were formed from bufotenine and the 6-hydroxy analogue, but with psilocine a deep blue colour appeared during incubation; this is analogous to the observations with the *Mytilus* enzyme. Measurement of the absorption spectrum of the blue oxidation product showed the same two maxima seen with the *Mytilus* enzyme (see Fig. 2).

Neither 5-hydroxyindole nor 4-, 5- or 7-hydroxytryptamine was oxidized by caeruloplasmin. In order to ensure that the lack of oxidation of these amino-acids was not due to a difference in *p*H optimum, measurements with 5-hydroxytryptophan as substrate were carried out at *p*H 5.0, 6.0 and 7.4. The absence of oxidation of the amino-acids is in contrast to what is seen with the *Mytilus* enzyme. Thus, the nature of the side chain is of importance in determining whether or not an indole derivative is oxidized by caeruloplasmin.

Like the gill plate enzyme, caeruloplasmin readily attacked both 4- and 7-hydroxytryptamine. Again, a deep blue colour appeared during oxidation of the 4-hydroxy compound, and the reaction proceeded at a rate considerably more rapid than with 5-hydroxytryptamine. In contrast to the *Mytilus* preparation, caeruloplasmin oxidized 7-hydroxytryptamine at nearly 10 times the rate seen with 5-hydroxytryptamine; the pigment formed again had a dark brown colour.

Table II lists the initial rates of oxidation of all substances tested, in % of that of 5-hydroxytryptamine. The table contains the results obtained with the *Mytilus* enzyme as well as with the pig plasma preparation.

In the blood plasma of a number of mammalian species there occurs an amine oxidase that acts on benzylamine and other amines (Blaschko, Friedman, Hawes, and Nilsson, 1959); such an enzyme is found in pig plasma. Dialysed pig serum slowly oxidizes 5-hydroxytryptamine. In the work of Blaschko *et al.* (1959) it was assumed that the oxidation of 5-hydroxytryptamine was catalysed by the amine oxidase present. However, it has now been found that in the purification of

TABLE II

RELATIVE RATES OF OXIDATION OF DIFFERENT SUBSTRATES BY THE HYDROXYINDOLE OXIDASE OF *MYTILUS* GILL PLATES AND BY PIG PLASMA CAERULOPLASMIN

The initial rate of oxidation of 5-hydroxytryptamine is taken as 100.

Substrate	Hydroxyindole Oxidase from *Mytilus* Gill Plates	Pig Plasma Caeruloplasmin
4-Hydroxytryptamine	520	700
5-Hydroxytryptamine	100	100
7-Hydroxytryptamine	100	1,100
Psilocine	145	230
Bufotenine	60	44
6-Hydroxy analogue of bufotenine	55	170
N-Methyl psilocine	120	—
N-Methyl bufotenine	78	—
4-Hydroxytryptophan	200	0
5-Hydroxytryptophan	50	0
7-Hydroxytryptophan	100	0
5-Hydroxyindoleacetic acid	82	—
5-Hydroxyindole ..	100	0
Paraphenylenediamine	150	6,000

caeruloplasmin the ability to oxidize 5-hydroxytryptamine could be separated from the benzylamine oxidase action; activity with 5-hydroxytryptamine always ran parallel to the ability of the fractions to oxidize paraphenylenediamine. In other words, the experimental evidence, to be described in detail elsewhere, suggests that the oxidation of 5-hydroxytryptamine by pig serum is brought about by the caeruloplasmin present, not by the amine oxidase.

Attempts were also made to study the effect of 5-hydroxytryptamine on the oxidation of paraphenylenediamine by caeruloplasmin. However, it was noted that a rapid oxygen uptake occurred when 5-hydroxytryptamine was added to paraphenylenediamine in the absence of caeruloplasmin; at the same time a deep purple colour appeared in the flask. This observation made it impossible to find out if the enzymic oxidation of paraphenylenediamine was affected by 5-hydroxytryptamine.

DISCUSSION

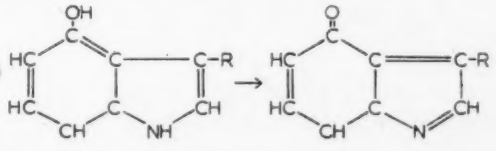
The experiments described have shown many similarities between the two enzyme preparations studied, but there are also minor differences. In a preliminary note, in which the action of *Mytilus* oxidase on the derivatives of 4-hydroxy and 6-hydroxyindole was described, the name "hydroxyindole oxidase" was proposed for the gill plate enzyme (Blaschko and Levine, 1960), because oxidation was not restricted to indole derivatives carrying a hydroxyl group in the 5-position. We can now say that both the *Mytilus* enzyme and caeruloplasmin will act on derivatives of 4-, 5-, 6- and 7-hydroxyindoles. There are some differences in substrate specificity: the *Mytilus* oxidase acted also on 4-, 5- and 7-hydroxytryptophan and on 5-hydroxyindole, none of which was attacked by the pig plasma enzyme. One is left with the impression that the range of substrates of the mammalian oxidase is narrower, a difference from the *Mytilus* oxidase probably due to specific differences in the two enzyme proteins.

The reactions catalysed by the two enzymes lead to the formation of the same coloured products. The chemical composition of these compounds has not been studied, but the fact that a similar blue colour is obtained when psilocine is oxidized by ferric ions suggests that the reaction involves an oxidation at the phenolic hydroxyl group. The colour reaction of psilocine with Keller's reagent (Hofmann *et al.*, 1959) may be similarly explained.

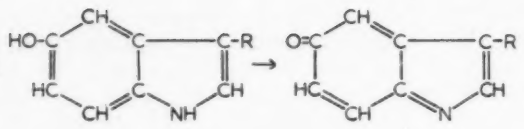
The possibility has already been discussed that the first attack of the *Mytilus* enzyme on the 5-hydroxyindoles results in the formation of a quinone-imine (Blaschko and Milton, 1960). It was therefore of interest to study the hydroxyindole derivatives which contain a phenolic hydroxyl in different positions of the indole nucleus. We have now found that 4-, 6- and 7-hydroxyindole derivatives are oxidized by the two oxidases.

A quinone-imine could also be formed from 7-hydroxyindoles, since in these compounds the phenolic hydroxyl group is in position ortho to the indole nitrogen, but the question arises whether the oxidation of substances carrying a hydroxyl group in positions 4- or 6- can be reconciled with the formulation previously given.

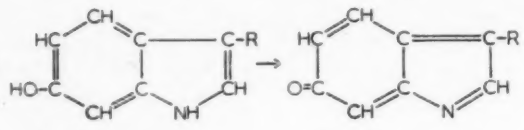
It is possible that the conjugated systems formed in the primary attack of the oxidases on 4- and 6-hydroxyindoles are different from those formed in the oxidation of the 5- and 7-hydroxyindoles. The reactions believed to occur are shown below:

(1) 

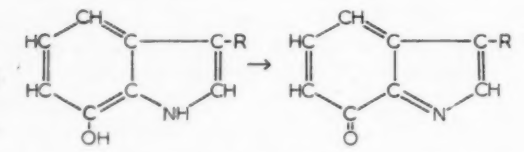
(2)



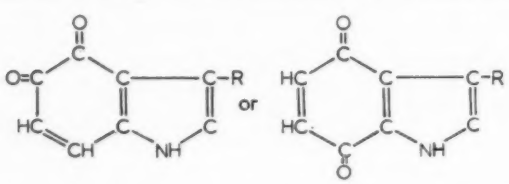
(3)



(4)



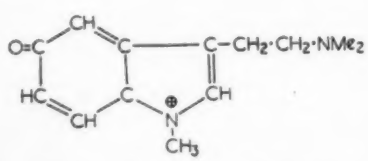
The formulation given for the 5-hydroxy compounds is that already discussed (Blaschko and Philpot, 1953; Blaschko and Milton, 1960). The oxidation of the 6-hydroxyindole derivative would also lead to a paraquinonoid structure, but the oxidation of the 4-hydroxyindoles would lead to an orthoquinonoid oxidation product. However, the reaction proposed under (1) is not the only one that has been considered. The blue oxidation product of psilocine may have a structure like this:



In this connexion it is of interest to note that Teuber and Staiger (1956) have prepared blue 4:5-diquinones by the action of potassium nitrosodisulphonate (Frémy's reagent) on hydroxyindoles.

The oxidation of 7-hydroxytryptamine (or of 7-hydroxytryptophan) would lead to the formation of an orthoquinone-imine.

The oxidation of the *N*-methyl derivatives of bufotenine and psilocine is of interest. If the formulation given under (2) is correct for bufotenine, the oxidation of the *N*-methyl derivative of bufotenine would be expected to lead to this product:



The structure of the oxidation product of *N*-methylpsilocine would probably be similar to the product of the enzymic oxidation of psilocine.

The evidence suggests that the mammalian and the molluscan enzymes catalyse the same reactions. Holmberg and Laurell (1951a) did not see any action of caeruloplasmin on monophenolic compounds. However, it has previously been pointed out that 5-hydroxytryptamine cannot be expected to behave like a typical monophenolic compound (Blaschko and Philpot, 1953; Blaschko and Milton, 1960); the presence of the indole nitrogen in position para to the phenolic hydroxyl group makes it comparable to a paradiphenol.

Caeruloplasmin is a copper-protein compound, and it is of interest that the properties of the molluscan oxidase are such that it too could be a metal-protein. This is an aspect that will have to await further purification of the *Mytilus* enzyme.

In *Psilocybe mexicana Heim* and related species of fungi, psilocine contributes only a small fraction of the total indole derivatives present (Hofmann and Troxler, 1959). Most of it is present as psilocybine, the phosphate ester of psilocine. Both psilocine and psilocybine are said to be hallucinogenic, but it seems worth while to consider the possibility that the phosphate ester exerts its central activity after being hydrolysed to psilocine and inorganic phosphate. At any rate, the biological inactivation of both substances may proceed *via* the free phenol.

This raises the question as to the physiological significance of the oxidation reactions here described. No precise answer to this question can at present be given. The catalytic action of caeruloplasmin on the catechol amines can easily be demonstrated *in vitro*, but it is still uncertain if such a reaction plays a part in their inactivation in the living animal. This has been fully discussed elsewhere (Blaschko, 1952b). The uncertainty arises from the fact that reducing agents, for example, ascorbic acid or sulphydryl compounds, immediately reduce the primary product of oxidation with regeneration of the free phenol. A similar mechanism might be operative in the oxidation of the hydroxyindoles. However, we do know that in both vertebrates and invertebrates oxidases of the type here studied are active on phenolic substances, in the formation of melanin. Enzymic processes of this kind may therefore occur in the living animal. It has been suggested that the indole derivatives might be possible pigment precursors in invertebrates (Blaschko and Milton, 1960), and similar considerations can be applied to the mammalian enzyme.

The oxidation of psilocine is so rapid that it is tempting to assume that the reaction studied is of importance in the biological inactivation of this compound. Whether the reactions here studied constitute an alternative pathway of catabolism of the 5-hydroxyindoles remains to be elucidated.

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